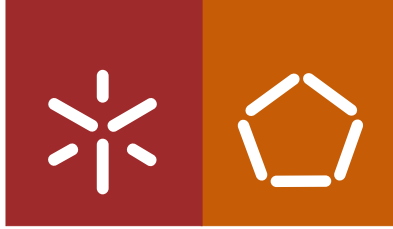


**Universidade do Minho**  
Escola de Engenharia

Catarina Cubo da Fonte

**Ecological shift of oral microbiota**



**Universidade do Minho**

Escola de Engenharia

Catarina Cubo da Fonte

## **Ecological shift of oral microbiota**

Dissertação de Mestrado  
Mestrado Integrado em Engenharia Biomédica  
Ramo de Engenharia Clínica

Trabalho realizado sobre a orientação da  
**Professora Doutora Mariana Contente Rangel  
Henriques**

Universidade do Minho  
e do

**Professor Doutor Wim Teughels**  
Katholieke Universiteit of Leuven

## DECLARAÇÃO

Nome: Catarina Cubo da Fonte

Endereço eletrónico: a55581@alunos.uminho.pt

Telefone: +351 963643519

Número do Cartão do Cidadão: 13767302

Título dissertação:

Ecological shift of oral microbiota

Alterações ecológicas da microflora oral

Orientadores:

Professora Doutora Mariana Contente Rangel Henriques

Professor Doutor Wim Teughles

Ano de conclusão: 2013

Designação do Mestrado:

Mestrado Integrado em Engenharia Biomédica

Ramo de Engenharia Clínica

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Universidade do Minho, \_\_\_\_/\_\_\_\_/\_\_\_\_

Assinatura: \_\_\_\_\_

## Acknowledgments

My first words are to my supervisors, Dr. Wim Teughels and Dr.<sup>a</sup> Mariana Henriques, I am especially grateful. They were always so understanding and available to help and meet with me. I want to express my thankfulness to Dr. Wim Teughels for the opportunity to integrate his investigation team in the Department of Periodontology of the Catholic University of Leuven. This work would not be possible without his ideas and advices. I specially acknowledge to Dr.<sup>a</sup> Mariana Henriques for the patience, understanding, guidance and motivation.

I want to express a special thanks to Gitte Loozen and Martine Pauwels. Gitte was untiring, I have to thank all the time she spent hearing my doubts and questions and explaining me. I will always remember all the availability, her advices and what she taught me. Martine was always helpful, especially with the molecular techniques I learned in the lab. She also had friendly words to my work, when my bacteria did not grow or when some techniques did not work. I will never forget her 'Allez' and complains about the weather. I also have to acknowledge to the laboratory people for the hospitality and sympathy, and the good working environment, mainly to Jesica and Mariana.

I would like to express a special word to my Erasmus mates (Joana Festa, José Sequeiros, Mariana Roriz, Pedro Morais, Pedro Vieira and Sandro Queirós) for all mishaps and experiences we lived together in a different country. Thank you to all Erasmus students I met during my time in Leuven. I also would like to thank to LUK singers for each music moments.

Another special word goes to my course friends, they were like a new family I found in a new city with which I grew up and I lived so good moments. Thank you for the fellowship and friendship. I also have to thank to people from the academic choir, CAUM, for each rehearsal and concert.

I also want to dedicate a special thanks to each person that encouraged me during thesis writing, for their support and motivation.

My last words must be addressed to my beloved mother and father. I wish to stress their unconditional love, confidence, support, help, encouragement, patience and understanding, without them this could not be possible. Thank you.

To my maternal grandparents,  
they would be proud of me.



## Ecological shift of oral microbiota - Abstract

The oral cavity is composed of several bacterial species living in a dynamic and complex ecosystem. Periodontitis and dental caries are two of the most prevalent oral diseases, nowadays. However, current treatments are not enough to fight these oral diseases, so alternative ways are required, as the use of prebiotics and probiotics, which are already being used in several fields.

The present work represents the study of the effect of a prebiotic compound (C7) on cariogenic bacteria in order to understand whether addition of this compound leads to a decrease of these bacteria. *Streptococcus mutans* and *Streptococcus sobrinus* were the cariogenic bacteria used in this study because these are the main cariogenic bacteria. A probiotic strain, *Streptococcus salivarius*, was also used in this study.

Firstly, quantitative Polymerase Chain Reaction (qPCR) was developed for *Streptococcus salivarius* in order to allow an accurate determination of its presence in microbial communities. The primers were chosen based on a conserved region of the dextranase gene of *S. salivarius* and the best combination of primers and probe concentration was determined. This quantification of this strain by this molecular technique was compared with microbial culturing presenting a linear relationship.

It was also intended, the find a selective medium for each of the species used, so different media were tested and TYCSB medium showed to be a good selective medium for *S. mutans* and *S. sobrinus*. However no selective medium was found for *S. salivarius*.

In order to determine the effect of the prebiotic compound, dual species experiment for each cariogenic bacterium and a probiotic species was carried out. Microbial culturing and qPCR were used for bacteria quantification and pH was also measured. For *S. mutans*, the main reduction was apparently due to the presence of *S. salivarius* and was not influenced by C7. For *S. sobrinus*, the verified reduction was the result of presence of *S. salivarius* with influence of C7, but no clear conclusions can be made about it.

In addition a Denaturing Gradient Gel Electrophoresis (DGGE) was performed to understand the effect of the prebiotic compound in saliva microbiota. The results demonstrated an ecological shift between different bacterial species present in saliva. So, *S. salivarius* seems to be in higher amounts when C7 is present while other species seem to be present in higher concentration when there is no C7.



## Alterações ecológicas da microflora oral - Resumo

A cavidade oral é um ecossistema dinâmico e complexo no qual diversas espécies vivem e interagem. Hoje em dia, a periodontite e as caries dentárias são duas das doenças orais mais prevalentes no mundo. No entanto, os tratamentos atuais não têm sido suficientes para responder a estas doenças, havendo necessidade de se utilizar outras alternativas. O uso de prebióticos e probióticos pode ser uma hipótese, tendo em conta que já têm vindo a ser utilizados noutras áreas.

Este trabalho representa o estudo do efeito de um composto prebiótico (C7) em bactérias cariogénicas, tentando perceber de que forma a sua presença permite levar à diminuição destas. Deste modo, foram utilizadas as principais bactérias cariogénicas (*Streptococcus mutans* e *Streptococcus sobrinus*) e também uma espécie probiótica (*Streptococcus salivarius*).

Assim sendo, foi desenvolvida uma metodologia de *Polymerase Chain Reaction* em tempo real (qPCR) para *S. salivarius*, de modo a possibilitar a sua correta quantificação em comunidades microbianas. A melhor combinação de concentrações dos *primers* e *probe* foi definida e a quantificação por este método foi comparado com a cultura microbiana, apresentando uma relação linear. A pesquisa de meios seletivos para cada uma das espécies usadas foi também realizada neste trabalho, pelo que foram testados vários meios. O meio TYCSB mostrou ser seletivo para ambas as espécies cariogénicas, contudo não foi encontrado nenhum meio seletivo para o *S. salivarius*.

Para determinar o efeito do composto prebiótico foi utilizado um modelo de espécies dual para cada bactéria cariogénica conjugando-a com a espécie probiótica. A quantificação destas estirpes foi feita através de cultura microbiana e qPCR e o pH foi também medido. Para o *S. mutans*, a principal redução verificada aparentou dever-se à presença do *S. salivarius* e não devido à influência do C7. Para o *S. sobrinus*, a redução que se verificou também resultou da presença do *S. salivarius* com uma ligeira influência do C7, embora estas conclusões não sejam muito claras.

Para perceber o efeito do C7 na saliva foi realizado uma DGGE (*Denaturing Gradient Gel Electrophoresis*), tendo os resultados demonstrado uma alteração ecológica entre as diferentes espécies presentes na saliva. Assim, *S. salivarius*, pareceu estar mais presente quando o C7 se encontra adicionado, enquanto outras parecem estar em maiores quantidades quando este composto não se encontra presente.





## Table of Contents

Acknowledgments .....	iii
Ecological shift of oral microbiota – Abstract .....	v
Alterações ecológicas da microflora oral – Resumo .....	vii
Table of Contents .....	ix
Abbreviations .....	xi
List of figures .....	xiii
List of tables .....	xvii
Chapter 1 – Introduction .....	1
1.1. Main objectives .....	3
1.2. General concepts .....	3
1.2.1. The oral cavity .....	3
1.2.2. Oral biofilms .....	4
1.3. Plaque-related diseases .....	6
1.3.1. Dental plaque and oral diseases .....	6
1.3.2. Periodontitis .....	7
1.3.3. Dental caries .....	9
1.3.4. Ecological plaque hypothesis .....	11
1.4. New treatments .....	15
Chapter 2 – Development of a new qPCR for <i>Streptococcus salivarius</i> .....	19
2.1. Introduction .....	21
2.2. Materials and Methods .....	22
2.2.1. Bacterial strains and culturing conditions .....	22
2.2.2. Design of qPCR <i>primers</i> and <i>probe</i> .....	22
2.2.3. Construction of the qPCR plasmid standard .....	23
2.2.4. qPCR optimization .....	23
2.2.5. Comparison between qPCR and microbial culturing .....	23
2.3. Results and Discussion .....	24
2.3.1. Design of qPCR <i>primers</i> and <i>probe</i> .....	24
2.3.2. qPCR optimization .....	25
2.3.3. Comparison between qPCR and microbial culturing .....	26
2.4. Conclusion .....	26

Chapter 3 – Effect of a prebiotic compound on cariogenic and saliva bacteria .....	27
3.1. Introduction .....	29
3.2. Materials and Methods .....	30
3.2.1. Bacterial strains and culturing conditions .....	30
3.2.2. Media testing .....	31
3.2.3. Growth in dual-species model .....	31
3.2.4. DNA extraction and quantitative Polymerase Chain Reaction (qPCR) ....	32
3.2.5. Saliva collection and preparation .....	33
3.2.6. DNA extraction, PCR-DGGE assay .....	33
3.3. Results .....	34
3.3.1. Selective medium for <i>Streptococcus salivarius</i> , <i>Streptococcus mutans</i> and <i>Streptococcus sobrinus</i> .....	34
3.3.2. Effect of a prebiotic compound on cariogenic bacteria .....	35
3.3.3. Effect of a prebiotic compound on bacteria present in saliva .....	42
3.4. Discussion .....	43
3.5. Conclusion .....	48
Chapter 4 – Conclusion and future work .....	51
Chapter 5 – References .....	55

## Abbreviations

*A. a.: Actinobacillus actinomycetemcomitans*

AER: aerobic conditions

ANA: anaerobic conditions

BHI: Brain-Heart Infusion

bp: base pair

C7: prebiotic compound

CFU: colony-forming units

CO<sub>2</sub>: carbon dioxide

DGGE: Denaturing Gradient Gel Electrophoresis

DMF: Decayed, Missing, Filled

DMFS: Decayed, Missing, Filled Surfaces

DMFT: Decayed, Missing, Filled Teeth

DNA: deoxyribonucleic acid

dNTP: deoxynucleoside triphosphate

EDTA: ethylenediaminetetraacetic

Eh: redox potential

EMA: ethidium monoazide

EPS: Extracellular Polymeric Substances

FAM: 6-carboxyfluorescein

FOS: fructo-oligosaccharides

GC content: guanine-cytosine content

GCF: Gingival Crevicular Fluid

GI tract: gastrointestinal tract

GOS: galacto-oligosaccharides

gtf: glucosyltransferase

IPS: Intracellular Polymeric Substances

KCl: potassium chloride

*L. fermentum: Lactobacillus fermentum*

*L. reuteri: Lactobacillus reuteri*

*L. rhamnosus: Lactobacillus rhamnosus*

*L. salivarius: Lactobacillus salivarius*

MgCl<sub>2</sub>: magnesium chloride

MM: Miminal Medium

MS: Mitis-Salivarius

MS-MUT: selective medium for *Streptococcus mutans*

MS-SOB: selective medium for *Streptococcus sobrinus*

OD: optical density

*P. g.*: *Porphyromonas gingivalis*

PCR: Polymerase Chain Reaction

PCR-DGGE: Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis

PMA: propidium monoazide

qPCR: Quantitative Polymerase Chain Reaction

R<sup>2</sup>: correlation coefficient

*S. mitis*: *Streptococcus mitis*

*S. mutans*: *Streptococcus mutans*

*S. oralis*: *Streptococcus oralis*

*S. salivarius*: *Streptococcus salivarius*

*S. sanguinis*: *Streptococcus sanguinis*

*S. sobrinus*: *Streptococcus sobrinus*

SD: standard deviation

TAMRA: 6-carboxytetramethylrhodamine

T<sub>m</sub>: melting temperature

TYC: Trypticase, Yeast, Cysteine

TYCSB: Trypticase Yeast Cysteine Sucrose Bacitracin

UV: ultraviolet

VBNC: Viable But Non-culturable Cells

w/v: weight per volume

## List of figures

Figure 1 - Oral biofilm formation. A. Pellicle formation. The acquired pellicle is a thin layer that consists of adsorbed organic molecules derived from the salivary glycoproteins attached to the tooth surface. B. Initial adhesion. Specific receptors allow the initial adhesion of bacteria to the pellicle. C. Maturation. Biofilm maturation results of interactions between later colonizers and early colonizers, previously attached in a cell-to-cell reaction (co-aggregation). D. Dispersion. Bacteria leave the biofilm and colonize a new site [2].	4
Figure 2 - Ecological plaque hypothesis and prevention of periodontal diseases. Gingival Crevicular Fluid (GCF). Redox potential (Eh) [10].	13
Figure 3 - Ecological plaque hypothesis and prevention of dental caries [10].	14
Figure 4 - Extended caries ecological hypothesis [20].	15
Figure 5 - PCR amplification for detection of the dextranase gene of oral streptococcal species by a PCR and a DNA probe (Ssal497T) with Ssal442F and Ssal615R primer pair: <i>S. mutans</i> (lane 1), <i>S. sobrinus</i> (lane 2), <i>S. salivarius</i> ATCC 7073 (lane 3), <i>S. salivarius</i> K12 (lane 4), <i>S. salivarius</i> clinical strain (lane 5), <i>S. salivarius</i> TOVE-R (lane 6). Negative control (lane 7) was made with physiological water.	25
Figure 6: Number of colony forming units (CFU) of <i>Streptococcus salivarius</i> (Y-axis) versus quantification of <i>Streptococcus salivarius</i> by quantitative Polymerase Chain Reaction (qPCR) (X-axis). Correlation coefficient: $R^2=0.9741$ .	26
Figure 7: Growth on Mitis-Salivarius agar plates (I) and on Trypticase Yeast Cysteine Sucrose Bacitracin (TYCSB) plates (II). (A): <i>Streptococcus salivarius</i> , (B): <i>Streptococcus mutans</i> and (C): <i>Streptococcus sobrinus</i> .	35
Figure 8: Number of colony forming units (CFU) of <i>Streptococcus mutans</i> in dual species model at 0 h, 24 h and 48 h: Growth of <i>S. mutans</i> testing the effect of C7 compound with (E) or without (D) the presence of <i>S. salivarius</i> and glucose with (C) or without (B) <i>S. salivarius</i> . The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and <i>S. salivarius</i> without addition of glucose (F). Standard errors of the mean (n=3)	

are represented by error bars. Significance ( $p < 0.05$ ) between the control (only *S. mutans*) and test series was determined using Student's t-test and are marked with \*. ..... 36

Figure 9: Number of *Streptococcus mutans* in dual species model at 0 h, 24 h and 48 h determined by quantitative Polymerase Chain Reaction (qPCR). The effect on *S. mutans* of C7 compound with (E) or without (D) the presence of *S. salivarius* and glucose with (C) or without (B) *S. salivarius*. The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and *S. salivarius* without addition of glucose (F). Standard errors of the mean ( $n=2$  for 0 h and  $n=3$  for 24 h and for 48 h) are represented by error bars. Statistically significant differences ( $p < 0.05$ ) between the control (only *S. mutans*) and test series were determined using Student's t-test and are marked with \*. ..... 37

Figure 10: Number of *Streptococcus salivarius* in *Streptococcus mutans* dual species model experiment at 0 h, 24 h and 48 h determined by quantitative Polymerase Chain Reaction (qPCR): *S. mutans* conjugated with *S. salivarius* and glucose (C), *S. mutans* together with *S. salivarius* and the C7 compound (E). The control was made with the cariogenic bacterium and *S. salivarius* (F). Standard errors of the mean ( $n=3$ ) are represented by error bars. Statistically significant differences ( $p < 0.05$ ) between the cariogenic bacterium (*S. mutans*) together with the probiotic bacterium (*S. salivarius*) with and without glucose are marked with \* and was determined using Student's t-test. .... 38

Figure 11: Number of colony forming units (CFU) of *Streptococcus sobrinus* in dual species model at 0 h, 24 h and 48 h: Growth of *S. sobrinus* testing the effect of C7 compound with (E) or without (D) the presence of *S. salivarius* and glucose with (C) or without (B) *S. salivarius*. The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and *S. salivarius* without addition of glucose (F). Standard errors of the mean ( $n=3$ ) are represented by error bars. Significance ( $p < 0.05$ ) between the control (only *S. sobrinus*) and test series was determined using Student's t-test and are marked with \*. .... 39

Figure 12: Number of *Streptococcus sobrinus* in dual species model at 0 h, 24 h and 48 h determined by quantitative Polymerase Chain Reaction (qPCR): *S. sobrinus* culture testing the effect of C7 compound with (E) or without (D) the presence of *S. salivarius* and glucose with (C) or without (B) *S. salivarius*. The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and *S. salivarius* without addition of

glucose (F). Standard errors of the mean (n=3) are represented by error bars. Significance ( $p<0.05$ ) between the control (only *S. sobrinus*) and test series was determined using Student's t-test and are marked with \*. ..... 40

Figure 13: Growth of *Streptococcus salivarius* in *Streptococcus sobrinus* dual species model experiment at 0 h, 24 h and 48 h with quantitative Polymerase Chain Reaction (qPCR): *S. sobrinus* conjugated with *S. salivarius* and glucose (C), *S. sobrinus* together with *S. salivarius* and the C7 compound (E). The control was made with the cariogenic bacterium and *S. salivarius* (F). Standard errors of the mean (n=3) are represented by error bars. Statistically significant differences ( $p<0.05$ ) between the cariogenic bacterium (*S. sobrinus*) together with the probiotic bacterium (*S. salivarius*) with and without C7 compound are marked with \* and was determined using Student's t-test. .... 41

Figure 14: PCR-DGGE analysis of effect of C7 compound in BHI and saliva after 24 h. BHI: Brain-Heart Infusion. C7: prebiotic compound. ANA: Anaerobic Conditions. AER: Aerobic Conditions. Black arrow: *S. salivarius* marker. Red rectangles: bands more clearly with the presence of C7 compound. Yellow rectangles: bands more clearly without the presence of C7 compound. .... 42





## List of tables

Table 1 – Comparison of the sequences of primers and probe designed for different strains of mutans streptococci .....	24
Table 2 – qPCR primers and probe: Guanine-Cytosine content (% GC), Melting temperature (T <sub>m</sub> )	24
Table 3: Conditions used in dual-species model growth with each one of cariogenic bacteria ( <i>Streptococcus mutans</i> or <i>Streptococcus sobrinus</i> ). Prebiotic compound (C7) .....	31
Table 4: Sequence and final concentrations of primers and probe used in quantitative Polymerase Chain reaction (qPCR) assay for <i>Streptococcus salivarius</i> , <i>Streptococcus mutans</i> and <i>Streptococcus sobrinus</i> .....	32
Table 5: pH values for <i>Streptococcus mutans</i> in dual species model at 24 h and 48 h: Assessment of pH variation of <i>S. mutans</i> culture testing the effect of C7 compound with (E) or without (D) the presence of <i>S. salivarius</i> and glucose with (C) or without (B) <i>S. salivarius</i> . The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and <i>S. salivarius</i> without addition of glucose (F) .....	38
Table 6: pH values for <i>Streptococcus sobrinus</i> in dual species model at 24 h and 48 h: Assessment of pH variation of <i>S. sobrinus</i> culture testing the effect of C7 compound with (E) or without (D) the presence of <i>S. salivarius</i> and glucose with (C) or without (B) <i>S. salivarius</i> . The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and <i>S. salivarius</i> without addition of glucose (F) .....	41



# CHAPTER 1

## Introduction



## 1.1. Main objectives

The aim of this work is the evaluation of the effect of a prebiotic compound (C7) stimulating *Streptococcus salivarius* to a mixture of cariogenic bacteria (*Streptococcus mutans* and *Streptococcus sobrinus*). The purpose is to analyze and discover whether addition of this compound leads to a decrease in the concentration of these cariogenic bacteria.

Moreover, the effect of this compound in saliva is also aimed in this work in order to test which bacteria are stimulated by this prebiotic compound.

The discovery of a selective medium for each bacteria used (*S. salivarius*, *S. mutans* and *S. sobrinus*) is also aimed to quantify these strains by microbial culturing. The development of a quantitative Polymerase Chain Reaction (qPCR) for *S. salivarius* is another goal of this work to make possible its quantification by this molecular technique.

## 1.2. General Concepts

### 1.2.1. The oral cavity

The oral cavity is a complex microbial ecosystem consisting of several bacterial species that interact with each other competitively and cooperatively in a not isolated or confined compartment within the human body [1]–[5]. The mouth comprises teeth, supporting tissues and oral mucosa [6]. Bacteria colonize different structures such as teeth, tongue and oral mucosa, and some bacteria are associated to the maintenance of oral health and balance with the host and the environment, while others are related to biofilm formation and to oral diseases [1], [2]. The resident microflora differs in composition according to surface and consists not only by Gram-positive and Gram-negative bacteria, but also by other species as yeasts [7], [8]. Several oral bacteria are associated to systemic diseases such as cardiovascular diseases and bacterial endocarditis [9].

The mouth has permanently shedding forces and organisms need to be firmly attached to avoid being washed away [8]. Saliva is a complex fluid produced by salivary glands that helps the mouth to get the optimal conditions for the growth of numerous microorganisms [6], [10], [11].

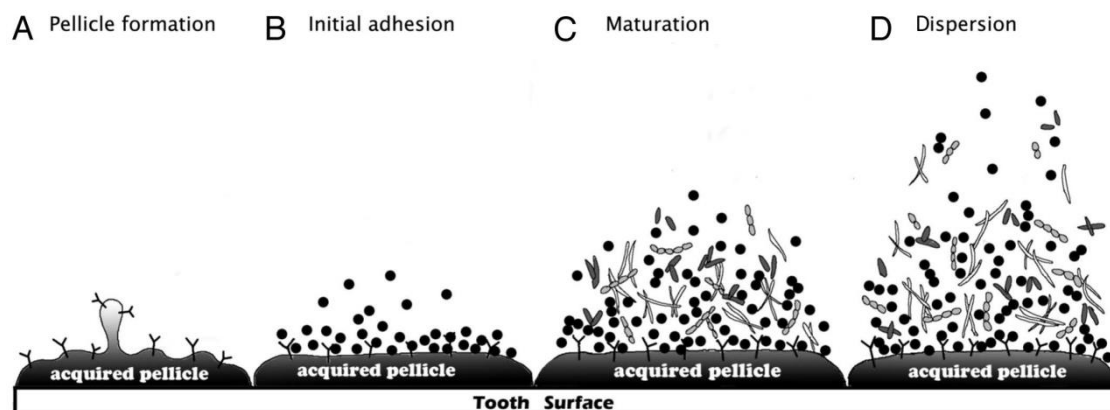
Saliva has several protective functions such as the production of a digestive enzyme or antibodies, keeping the warm, clean and moist conditions (maintaining the normal pH of the oral microflora values around 6.75-7.25 and the temperature around 35-36 °C) or helping the speech [6], [8], [10]–[12]. Saliva has buffering capacity to restore the pH and is a primary source of

carbohydrates, peptides and amino acids associated to clearance of fermentable sugars in mouth [7]. Saliva influences the ecology of the mouth with its ionic composition and organic components such as glycoproteins and proteins [10].

### 1.2.2. Oral biofilms

Oral biofilms are aggregates of microorganisms attached to a surface or to each other creating a dynamic and complex multispecies community called dental plaque leading to oral disorders such as periodontitis or dental caries [1], [2]. “Animalcules” were observed for the first time in gingival tooth scrapings by Antoine van Leeuwenhoek with a microscope [2], [13], [14].

Bacteria in biofilm form and act like a community and present some specific characteristics such as complex interspecies interactions, surface attachment, extracellular matrix of polymeric substances [1]. On the other hand, microorganisms that are free-floating and not attached are called planktonic cells and their characteristics differ from biofilms, which consist of with glycocalyx matrix and bacterial cells [2]. Planktonic cells can attach directly to surfaces of the oral cavity or to bacterial cells already colonized [1].



**Figure 1 - Oral biofilm formation.** A. Pellicle formation. The acquired pellicle is a thin layer that consists of adsorbed organic molecules derived from the salivary glycoproteins attached to the tooth surface. B. Initial adhesion. Specific receptors allow the initial adhesion of bacteria to the pellicle. C. Maturation. Biofilm maturation results of interactions between later colonizers and early colonizers, previously attached in a cell-to-cell reaction (co-aggregation). D. Dispersion. Bacteria leave the biofilm and colonize a new site [2].

Biofilm formation is a natural and highly dynamic process (Figure 1). It begins with the attachment of acquired pellicle by specific extracellular proteinaceous components (adhesins) crucial for the initiation of biofilm formation [2], [3], [15]. This pellicle is an acellular proteinaceous film, a thin layer of adsorbed organic molecules originates from the salivary glycoproteins, phosphoproteins and lipids attached to a clean tooth surface and consists of several components as glycoproteins, enzymes and other molecules [1]–[3], [8]. The mechanisms involved in acquired pellicle formation include long-range forces (Coloumb

interactions, van der Waals forces and dipole-dipole interactions), medium-range forces (hydrophobic interactions) and short range forces (covalent bonds, electrostatic interactions, hydrogen bonds, ionic interactions and Lewis acid-base interactions) and are based on Gibbs law of free enthalpy [2].

The second phase consists of initial adhesion of bacteria to the pellicle, which is important for oral bacteria interactions with host molecules allowing the connection between bacteria and receptors [1]–[3]. There are specific receptors in the pellicle on the tooth surface that allow bacterial binding based on a recognition system [8]. The mechanisms involved in the early attachments are the result of random bacterial movement and are based on electrostatic attractions or physical attachments and later on chemical forces, which include hydrogen bonds, hydrophobic interactions, calcium bridges, van der Waals forces, acid-base interactions and electrostatic interactions [2], [3], [8], [13]. This early attachment is weak and reversible and some bacteria previously attached may leave the tooth surface due to specific and non-specific molecular interactions involved on it. Early colonizers are pioneer species that attach the tooth surface mainly members of the genera *Actinomyces* and *Streptococcus* [2], [3], [7], [13].

The third phase comprises the attachment and biofilm maturation [2]. The attachment of colonizers is made through salivary glycoproteins by connection to early colonizers, previously attached to cells surfaces [1], [2]. Late colonizers include Gram-negative anaerobes, as for example *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Treponema* spp. [2], [8]. The attachment of later colonizing bacteria is a cell-to-cell reaction mediated by adhesin-receptor interactions called co-aggregation [1], [2], [8]. This specific process is very complex because bacteria can only aggregate with specific bacteria according to polysaccharide recognition and not with any random bacteria. When bacteria attach to the pellicle by specific interactions, extracellular polymeric substances (EPS) excretion begins and attachment becomes stronger and irreversible [2], [8], [13]. EPS is the major component of biofilms surrounding bacteria present in biofilm establishing its structure, promoting bacterial accumulation to the tooth surface, providing a communication medium between bacteria, and promoting biochemical and physiological changes in the matrix of the biofilm [2], [13], [16]. EPS are largely insoluble and biosynthetic polymers such as polysaccharides, proteins, nucleic acids and phospholipids [13]. A mature biofilm has different microbial components from the initial biofilm [2]. The mature biofilm is a stable situation that can enhance the resistance to antibiotics due to its complex structure and multispecies composition, when compared to planktonic cells [1], [2].



The last phase in biofilm formation is the dispersion of biofilm cells and colonization because bacteria leave the biofilm by erosion, sloughing, and seeding and so, they can spread to colonize a new site [2]. This detachment is due to limited nutrients on biofilm requiring a new site with more nutrients to grow and due to limitation of biofilm development (bacteria are better protected against fluid shear force of saliva on rough surfaces) [2], [3].

Microorganisms in dental biofilm can cooperate and also compete with each other [1], [2]. The bacteria present in biofilm maintain equilibrium with the host through microbial cell-cell interactions helping the community dynamics [1], [3], [17]. From all oral bacteria, *Streptococci* are Gram-positive that have the strongest ability to produce several kinds of bacteriocins such as mutacins (lantibiotics and non-lantibiotics) [1], [2], [10]. Bacteriocins are non-specific proteins produced derived of ribosomal synthesis of cationic peptides with antimicrobial activity [1], [2], [4]. Quorum sensing system is a chemical communication process among bacteria characterized by the production of signal molecules, transport, sense and control of bacterial growth [1], [2].

Moreover, some commensal bacteria present in the dental plaque are able to exclude some pathogens and allochthonous bacteria by production of antimicrobial substances or competition for nutrients [1], [3], [17]. However, the accumulation of dental biofilms also modifies the bacterial composition leading to oral diseases such as periodontitis or dental caries [1]. This modification is characterized by a shift in oral biofilms from Gram-positive bacteria to Gram-negative anaerobic rods.

So, the biofilm results of the attachment of the bacterial cells to a clean surface (tooth surface) forming an acquired pellicle, followed by accumulation and multiplication of bacteria resulting in the colonization and maturation of biofilm [15].

## 1.3. Plaque-related diseases

### 1.3.1. Dental plaque and oral diseases

Dental plaque is a dynamic microbial ecosystem in which several Gram-positive and Gram-negative bacteria, that interact with each other through microbial interactions, grow as a biofilm maintaining a dynamic stability stage - microbial homeostasis [7]–[9], [18]–[20]. However, changes and imbalances in the oral microflora can occur and the microbial homeostasis breaks down allowing the development of plaque-related diseases due to an enrichment of pathogens and a reduction of beneficial bacteria within the microbial community [4], [7], [19]. Oral diseases are the result of a shift in the balance of the resident microbiota [21].

Periodontal diseases are infections of the supporting tissues of the teeth that result of an inflammatory response disturbing the harmonious relationship in the oral cavity [22]–[24]. Periodontal diseases are the most common infection diseases in the world and remain an important health problem associated to tooth loss in adults [4], [24]–[26]. These diseases are characterized by an increase of obligatory anaerobic bacteria as Gram-negative proteolytic species [10].

Gingivitis is a reversible infection of the soft tissue of the mouth that does not destroy the periodontal tissues [22]. Gingivitis results from accumulation of plaque triggering an inflammatory response and increasing the gingival crevicular fluid [7]. Periodontitis is a polymicrobial infection due to colonization of hard and soft surface tissues in the oral cavity that can result in attachment loss and destruction of alveolar bone and eventually tooth loss [24], [27]–[29]. So, periodontitis is a more severe stage of the infection that results of the evolution of gingivitis [24].

Dental caries is an infectious disease due to bacterial action resulting of a process of demineralization of enamel crystals and dentin by acids derived from interactions of specific bacteria with sugars of the dental plaque leading to tooth destruction [30]–[33].

Dental diseases are one of the most prevalent diseases nowadays with high treatment costs [10].

### ***1.3.2. Periodontitis***

The periodontium is a set of tissues that supports the tooth and can be divided in gingiva, cementum, alveolar bone and periodontal ligament [33], [34]. Gingiva is the soft tissue that covers the mouth. Cementum is a specialized calcified and hard tissue that covers the root of the tooth. The alveolar bone is the bone in the jaw that supports and protects the teeth, and the part of the maxilla that helps the resistance of mastication. The periodontal ligament is a group of soft and connective tissue fibers that makes the connection between the tooth attachment and the cementum [24], [33], [34]. The functions of periodontium are the support and the attachment of the tooth to the bone of the jaw, and the protection and the resistance of the tooth to the mastication forces [24], [33], [34].

The pathogenic microorganisms involved in oral biofilm originating periodontitis are principally gram-negative pathogens such as *Porphyromonas gingivalis* (*P. g.*), *Prevotella intermedia*, *Fusobacterium nucleatum* and *Actinobacillus actinomycetemcomitans* (*A. a.*) [23], [24], [28]. However, these microorganisms are not in high number in initial lesion, but the

number increase with the development of disease [35]. The oral biofilm with pathogenic microorganisms is the main etiological factor of periodontitis [24]. For instance, *A. a.* is a gram-negative bacterium that is indigenous of the oral cavity [25], [36]. *A. a.* can induce the periodontal disease by colonizing tooth surface with adhesins but it is also associated to systemic infection as endocarditis. On the other hand, *P. g.* is an anaerobic, non-motile and non-sporulating Gram-negative rod able to colonize the gingival sulcus and also the periodontal pocket [14], [37]. Virulence factors of *P. g.* help this species to survive in adverse conditions of growth as periodontal pocket.

The aim of therapy and treatment for patients with periodontitis is to halt the progression of the disease removing the inflammation and reducing the periodontopathogens from the subgingival area [23], [24], [27]. Traditional treatment involves scaling and root planning conjugated with antibiotics [23], [24], [26], [27].

Scaling and root planning is a surgical therapy that consists in removing supra- and subgingival plaque and the plaque from the root surfaces of the teeth preventing the progression of periodontitis [14], [24]. The most effective method in scaling and root planning used is called periodontal debridement and aims to stimulate the regeneration of lost and damaged tissues and to reduce deep probing depth [24], [38]. This technique has several benefits such as the reduction of clinical inflammation, disease progression and probing depth, gain of clinical attachment, and microbial shifts of oral cavity to an oral microflora with less pathogen. However, scaling and root planning is technically difficult to perform due to some mechanical limitations and time consuming [24], [38]. Moreover, this technique is disagreeable for patients and some bacteria present can persist and recolonize [24], [27], [38].

The use of antibiotics is an adjunctive therapy to treat periodontitis [24]. The application of antibiotics can be systemic or local depending on severity of periodontitis. The most commonly systemic antibiotics used are tetracycline, ciprofloxacin, metronidazole and penicillins [24]. This type of pharmacologic administration is easy to perform, but is also associated to resistance of bacterial species. On the other hand, local delivery gives site specific and therapeutic level at the site of infection allowing localized treatment areas [24]. Tetracyclines, metronidazole and chlorhexidine are drugs used for local delivery. The disadvantages are associated to their cost due to successive treatments and in the inconvenience by changing oral hygiene habits [26]. However, this is not a definitive solution because (re)colonization of the periodontal pockets and

resistance to antibiotics can occur, which is more difficult with bacteria within biofilm [4], [23], [24], [27], [29].

Periodontopathogens can also enter the blood stream triggering new infections [24]. For this reason, periodontal diseases have been investigated in order to verify their association to other diseases. Current research supposes periodontal diseases increase cardiovascular diseases [22]. Genetic factors such as age, hypertension and diabetes or environmental factors as diet, stress or cigarette smoking are tested risk factors associated to cardiovascular diseases. Periodontopathogens have been found in the association of etiology in cardiovascular diseases [22].

### ***1.3.3. Dental caries***

Dental caries is one of the most common diseases affecting humans and one of the most prevalent chronic diseases, characterized by a very slowly progression in the majority of individuals and becoming the most expensive part of the body to treat [18], [30], [39], [40]. Pain, localized destruction of the hard tissues, tooth destruction and tooth loss and impaired quality-of-life are some consequences of untreated caries [18], [41].

When a biofilm on tooth surface is able to attach, grow, develop and mature, the process of formation of dental caries can initiate and progress, leading to the loss of mineral from the tooth and the localized destruction of the tooth [8], [40], [42]. Although the presence of biofilm is necessary to develop dental caries, not all biofilms lead to dental caries, i.e., the amount is not enough to trigger this process, and in this case teeth can be healthy covered by biofilms [40].

Consumption of fermentable carbohydrates are the key environmental factors involved in initiation and development of dental caries [7], [10], [16]. Sucrose is the most cariogenic dietary carbohydrate because it is fermentable and used as a substrate for extracellular glucan synthesis by glucosyltransferases from *mutans streptococci* and the synthesis of extracellular (EPS) and intracellular (IPS) polyssacharides in the dental plaque [16]. Low pH environment promotes a change in the resident oral plaque, while EPS promote changes in the composition of matrix of the biofilm [16]. EPS such as glucosyltransferases and fructosyltransferases help bacterial adherence and accumulation on tooth surface and create biochemical and structural changes in the matrix of biofilm, such increase the porosity. IPS such as glycogen-like help to maintain a low pH in the matrix of dental plaque and to exposure organic acids to tooth surfaces. Then, sugar can easily diffuse into the biofilm and decreasing local pH by microbial catabolism [16]. Furthermore, sucrose is able to reduce the concentrations of the most important ions in

maintaining the mineral equilibrium between the tooth and the oral environment (calcium, inorganic phosphorus and fluoride) [16].

An increase of sucrose-rich diet and carbohydrates metabolism promotes acidification creating conditions for 'low-pH' *non-mutans streptococci* species grow, increasing the risk of dental caries [10], [16], [20]. Acid production exposure the dental plaque continuously under the critical pH for demineralization of tooth surfaces leading to net mineral loss and chemical dissolution [10], [16], [18], [20], [30]. This destruction can affect enamel, dentin and cementum [40], [42], [43]. Enamel and dentin are part of the tooth. Enamel is a hard, inert and acellular tissue that covers the crown and it is also the most highly mineralized tissue found in the body. Dentin is a hard, elastic, resilient, sensitive, connective and avascular tissue that supports the enamel [6]. Consequently, acid production by oral bacteria due to sugar metabolism decreases environmental pH. So, dental caries is an endogenous disease that consists of metabolic events in dental biofilms resulting in an imbalance in the equilibrium between biofilm fluid and tooth mineral [10], [18], [20], [42]. As a consequence, acid production increases, environmental pH decreases and chemical composition of dental plaque shifts to a predominantly Gram-negative bacteria such *mutans streptococci* and *lactobacilli* [10], [18]–[20].

The traditional detection of dental caries is made by visual examination (white opaque lesions as a consequence of enamel translucency) and radiographs [41], [43]. Recently, other methods have been developed, e.g. methods based on fiber-optics, fluorescence or electrical impedance [41].

It has been used the DMF index to quantify caries, where D is for decayed teeth, M is for teeth missing and F is for teeth previously filled [44]. This index can be applied to teeth entirety (DMFT) or to all surfaces of the teeth (DMFS) [44]. However, the application of DMF index has been decreasing nowadays, except in the quantification of treatment received, mainly due to modern preventive and restorative technology [44].

Several microorganisms are involved in the formation of dental caries. These microorganisms should be able to produce acid and to tolerate a low-pH environment [18].

*Non-mutans streptococci* and *Actinomyces* species are present in high levels at the initial stage of plaque formation, probably due to adhesins which facilitate their adhesion to proteins and sugar chains of acquired pellicle on tooth surface [18], [20], [35]. These species can acidify the environment through degradation of carbohydrates creating acidic and anaerobic conditions [35]. *Non-mutans streptococci* strains such as *Streptococcus sanguinis*, *Streptococcus oralis* and

*Streptococcus mitis* are the initial colonizers involved and *mutans streptococci* are present in low level [18], [20]. When dental caries is developed, the most common species present are *Actinomyces* and *Streptococcus* [20].

*Mutans streptococci* are a group of microorganisms (such as *Streptococcus mutans* and *Streptococcus sobrinus*) able to grow and develop at conditions of high sugar and low pH [7], [10], [18], [19]. *Mutans streptococci* are highly acidogenic and aciduric species able to produce water-insoluble extracellular glucan from sucrose by glucosyltransferase promoting bacterial adhesion to tooth surface and to other bacteria [18], [20], [35]. Although *mutans streptococci* are associated to dental caries, the disease can occur in the absence of these microorganisms [19]. *Mutans streptococci* are the major pathogens involved in dental caries formation (specially related with sucrose-rich diet) due their aciduric and acidogenic characteristics [18], [20]. *S. mutans* is the main etiological agent of human dental caries [17]. Additionally, *S. sobrinus* is also associated with the formation of dental caries [39]. The risk of transmission from mother to child is another etiological factor of dental caries [45].

An invasive intervention in clinical dentistry is an operative way involved on caries treatment [46]. Vaccination, gene therapy or antimicrobial treatment are several ways to control dental caries, however elimination of specific bacteria responsible for caries is not an effective method because these bacteria are essential for mouth equilibrium [20]. An alternative is the use of probiotics to treat caries infection through interfere on oral colonization of cariogenic bacteria [5].

*S. mutans* and *S. sobrinus* are also associated with non-oral infections [39]. Diabetes can also be related to dental caries due to salivary dysfunction [12].

#### 1.3.4. Ecological plaque hypothesis

The mouth comprises surfaces such as mucosal surfaces and teeth (non-shedding surfaces) with different oral microflora. Some of these places, for example, teeth, allowing the attachment and growth of bacteria which leads to the dental plaque and later to disease, e.g., periodontitis or caries [10].

The resident oral microflora is characterized by a dynamic relationship with inter-microbial and host-microbial interactions. This microbial homeostasis leads to the stability of microflora [10]. Alterations between microbial ecosystem and host tissue are responsible for initiation of oral diseases [35].

The resident oral microflora has endogenous proteins and glycoproteins (mucins) as the main sources of carbon and nitrogen. Any change in the environment increasing oral pathogens within the microbial community will cause an imbalance in the microflora [10]. Changes in the nutrient status at the site, the diet, the dentition and radiation therapy are possible causes for alterations on oral microflora [10].

In a healthy situation, dental plaque results from a biofilm formation: conditioning film, early colonizers, attachment and colonization, later colonizers, co-aggregation and a microbial community [10]. In case of periodontitis, there is an increase of levels of obligatory anaerobic bacteria as a result of development of an inflammatory host response [10]. This increase will lead to inactivation of host proteins. In the case of dental caries, there is an increase on levels of acid-tolerating bacteria, principally *mutans streptococci* and *lactobacilli*, which will lead to demineralization of enamel as a result of development of an infection [10]. This increase in acidogenic and aciduric bacteria will lead to a decrease in pH by metabolizing dietary sugars to acid, creating an optimal growth conditions for these species.

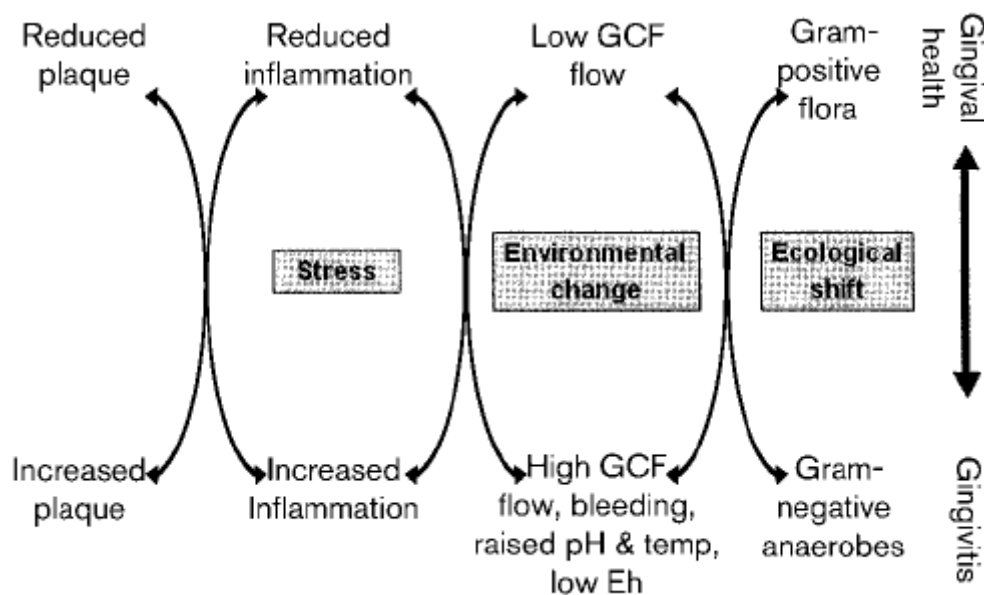
There are two hypothesis to explain the different bacteria species present in diseased and healthy sites [10], [19]. The 'specific plaque hypothesis' proposes that only a small percentage of organisms present in dental plaque are actively involved in disease [7], [10], [19]. This hypothesis focuses the treatment only against microorganisms responsible for the disease [19]. However, caries can appear in the absence of typical etiological agents and these species can be present without caries lesion development [7], [10], [18].

On the other hand, the 'non-specific plaque hypothesis' proposes that interactions between bacteria present in dental plaque and the host can lead to a disease situation [7], [10], [19]. So, dental caries and processes associated to their development can be controlled [18].

The 'ecological plaque hypothesis' is an alternative explanation to define the relationship between dental plaque bacteria and the host in health and disease resulting from the combination of specific and non-specific plaque hypothesis [7], [10], [16], [18], [19]. This hypothesis is an ecological and dynamic model based on changes in microbial and environmental dynamics (key factors) in oral microflora leading to the development of oral diseases [7], [10], [35].

In case of periodontal diseases (Figure 2), putative periodontal pathogens exist on healthy sites in a small proportion, but when plaque biomass accumulates, an inflammatory host response is triggered [10]. This results on the increase of flow of gingival crevicular fluid (derived

from blood plasma and is rich in nitrogenous compounds as peptides, amino acids and proteins) and alteration of local nutrients status and pH will change to a neutral value [7], [10], [35]. The consequences are an increase in pH (to a neutral value), a decrease in redox potential (Eh) and an outgrowth in proteolytic, anaerobic and asaccharolytic Gram-negative bacteria [7], [10]. The microbial shift of periodontitis consists of an enrichment of anaerobic obligatory and proteolytic bacteria (such as *Porphyromonas gingivalis*) due to increase of gingival crevicular fluid. This results from host defense induced by colonization when bacteria promote a neutral pH environment and increase Gram-negative and unculturable bacteria in the mouth [21], [35].



**Figure 2 - Ecological plaque hypothesis and prevention of periodontal diseases.** Gingival Crevicular Fluid (GCF). Redox potential (Eh) [10].

In case of dental caries (Figure 3), potentially cariogenic bacteria exist at neutral pH in a low level (clinically insignificant) [10]. A low-sugar diet promotes a stable plaque microflora and demineralization and remineralization are in equilibrium [7], [10]. However, with an increase in the frequency of fermentable carbohydrates consumption, pH will decrease and acid-tolerating bacteria will proliferate, stimulating demineralization [10], [19]. This situation will shift the subgingival microflora from mainly Gram-positive bacteria to a more cariogenic resident plaque microflora with high levels of anaerobic, asaccharolytic and obligatory Gram-negative organisms, the best adapted to high sugar and low pH, such as *mutans streptococci* and *lactobacilli*, on oral cavity [7], [10], [16]. The microbial shift of dental caries are characterized by increase of population of aciduric bacteria (*mutans streptococci* and *lactobacilli*) and enrichment of cariogenic potential of supragingival plaque [21], [35].



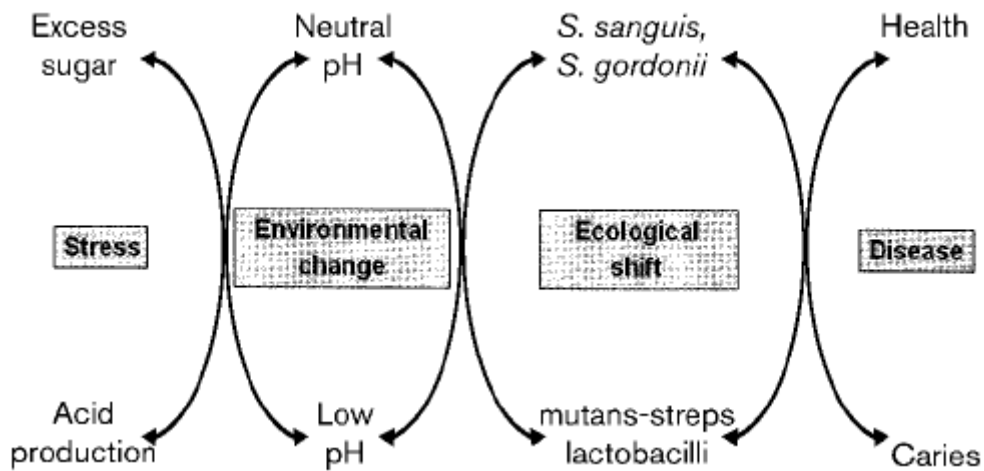


Figure 3 - Ecological plaque hypothesis and prevention of dental caries [10].

Prevention strategies depend on type of dental disease but the main idea is interfering on factors responsible for diseases shifting to a health situation [7], [19].

In case of periodontitis, the site should be less anaerobic and the flow of gingival crevicular flow would be reduced in order to avoid growth of putative pathogens [7], [10]. Anti-inflammatory and antimicrobial agents reduce gingival crevicular fluid and growth of some periodontopathogens becomes restrict according their essential nutrients. Oxygenating and redox agents are an alternative to create an incompatible environment for growth of obligatory anaerobes when the redox potential of periodontal pocket can be raised to create these conditions [7]. Methylene blue is a redox dye able to prevent the growth of obligatory anaerobes by the increase of redox potential and decrease of gingival crevicular fluid [7], [10].

In the case of dental caries, the actions are based on inhibition of plaque acid production and consumption of non-fermentable sugar compounds [10]. Fluoride is able to enhance remineralization and acid resistance of enamel. Antimicrobial agents as chlorhexidine reduces the impact of pH changes and demineralization, promoting mechanical cleaning of plaque [7], [10]. Other alternative sweeteners, also known as sugar substitutes, act as promoter of remineralization of enamel and stimulate saliva flow when there is no significant acid production [7], [10].

An extended caries ecological hypothesis (Figure 4), based on the ecological hypothesis, proposes three reversible stages in the caries process to explain the relationship between the composition of the dental plaque and dental caries process, conjugating microbiological, biochemical, ecological and clinical perspectives [18], [20]. Dynamic stability stage is composed mainly by non-mutans bacteria (*non-mutans streptococci* and *Actinomyces*) promoting a natural

pH cycle for development, growth and equilibrium in the oral cavity [18], [20]. These bacteria can produce acids from sugary foods and these acids can demineralize enamel, but the equilibrium is easily achieved by changes between mineral net gain and mineral net loss helping demineralization and remineralization balance. This situation stimulates selection and increase of 'low-pH' non-mutans bacteria and microbial acid-induced adaptation creating an acidogenic stage [18], [20]. However, if acidification steps are rarely, balance will shift to net mineral gain tending to remineralization. The acidic environment increases aciduric bacteria allowing lesion development and net mineral loss creating an aciduric stage [18], [20].

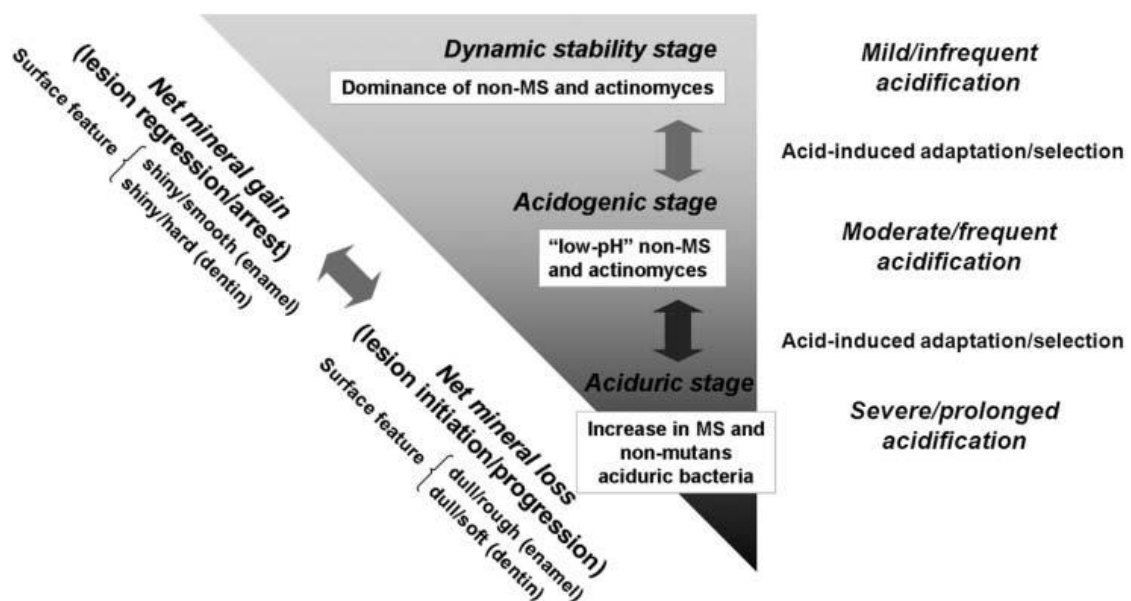


Figure 4 - Extended caries ecological hypothesis [20].

## 1.4. New treatments

As current treatment options are not solving the problem of oral disorders, specially due to antibiotic resistance, other options have to be investigated [21], [47]. Included in these new options are probiotics and prebiotics, which are emerging in diverse fields, as oral health.

Probiotics are defined as live microorganisms, which when administered in certain quantities, have health benefits on the host (humans and animals) [4], [5], [21], [46], [48], [49]. First definition for probiotics was introduced by Lilly & Stillwell in 1965 and then several alterations were made [5], [50]. Probiotics are characterized by beneficial effects (immune stimulation, immune modulation of host defenses, anticarcinogenic effects, antidiabetic characteristics, cancer prevention, ...), production of antimicrobial substances (depending on pH, catalase, proteolytic enzymes and temperature), adhesion to the mucosa, degradation of toxins,

improvement of colonization resistance, and competitive exclusion mechanisms [4], [46], [47], [49]–[51].

The main field of research of probiotics is the gastrointestinal (GI) tract and they are already being used in the GI tract (particularly the colon, a very heavily colonized site) to restore numbers of beneficial bacteria and decrease numbers of pathogenic bacteria [5], [21], [46]–[48], [51]. *Bifidobacteria* and *lactobacilli* are the most commonly probiotic species used such as *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Bifidobacterium bifidum* or *Bifidobacterium infantis* [5], [21], [46]–[48], [51].

Probiotics require *in vitro* tests and substantiation of efficacy with human trials before their use on humans in order to guarantee their beneficial effects [4].

In recent years use of probiotics has also been demonstrated for urogenital infections, atopic disease, voice prostheses and in the dental field [4], [5], [21], [46], [47].

An ‘oral probiotic’ needs to be able to adhere and colonize surfaces in the oral cavity such as hard non-shedding surfaces [4], [27], [46]. Oral probiotics should also not be able to ferment sugars, otherwise pH will decrease and caries will develop [4].

Some *lactobacilli* strains such as *L. fermentum*, *L. salivarius* and *L. rhamnosus* are used in dairy products (a way for probiotic administration), others are present in resident oral microflora [46]. *L. rhamnosus* GG has inhibitory activity against cariogenic *streptococci* [5], [47]. *Lactobacillus* species are also able to produce inhibiting substances avoiding and preventing adhesion and colonization of pathogenic bacteria [46]. For example, consumption of yogurt containing *L. reuteri* reduces *S. mutans* [46]. This reduction is verified during the period of yogurt consumption and some days after cessation of consumption, thereby studies are required to analyze caries-inhibiting effect after the probiotic administration [46], [47]. A reduction in *mutans streptococci* was also detected with *Bifidobacterium* DN-173 010 consumption [46]. *L. reuteri* is also able to reduce gingivitis [46]. *Lactobacilli* and *bifidobacteria* can inhibit growth and colonization of periodontopathogens to hard and soft tissues and can inhibit cariogenic *streptococci* [4], [47].

The best effect on the oral microbiota was seen when working with probiotic indigenous oral bacteria. Indigenous bacteria are already present in the oral microflora, do not need adaptation and do not change with intervention or disease [4], [50].

One example of such a bacterium is *Streptococcus salivarius* [46]. *S. salivarius* already demonstrated inhibitory effect on volatile sulfur compounds by competition for colonization sites

against other species [46]. This bacterium is one of the early colonizers of epithelial surfaces of the oral cavity [21].

An ideal way to stimulate the indigenous beneficial microbiota would be by prebiotics. Prebiotics are non-digestible food ingredients (substances or nutrients) that can beneficially affect, by stimulation, the growth and/or the activity of some bacterial species of the host [47]–[51].

To be considered as prebiotics, a dietary substrate needs to: be resistant and available in order to be used as fermentation substrate; be selective for beneficial bacteria; induce beneficial effects within the host by prebiotic fermentation [51], [52].

Prebiotics are non-digestible carbohydrates such as oligosaccharides [51]. Oligosaccharides are soluble short-chain polysaccharides with low degree of polymerization and can be found in fruits and vegetables and also produced by hydrolysis of polysaccharides. Lactulose, lactosucrose, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), gluco-oligosaccharides or xylo-oligosaccharides are some examples of oligosaccharides with prebiotic potential [21], [51], [52]. Prebiotics are often studied for application on GI tract, but other studies should be carried out to define their applicability into other areas [52].

Prebiotics produce protective metabolites, increase mineral absorption and reduce the risk of cancer, e.g., colon cancer [49], [51]. Prebiotics stimulate beneficial components proliferation in the microflora, specially components with probiotic characteristics such as *lactobacilli* and *bifidobacteria* [21], [49], [52]. The increase of these beneficial bacteria improve resistance to pathogenic bacteria due to production of natural antibiotics with inhibitory properties and antimicrobial effects [51].

Different techniques have been developed to enumerate bacteria such as Polymerase Chain Reaction (PCR) or Denaturing Gradient Gel Electrophoresis (DGGE) due to their applicability to culturable as well unculturable cells verifying that species present in disease are already on microflora in an healthy stage but at low numbers [21], [52].

The combination of pre and probiotics could be another point of interest for prevention and treatment to improve oral health [4]. A mixture that contains pre and probiotics and that beneficially affects the host is known as symbiotic [5], [50]. Previous research already demonstrated the inhibitory effect of pre and probiotics for head/neck, oral and respiratory tracts, pancreas and liver, and kidney, bladder and vagina [48]. Regarding to the oral cavity, the inhibitory effect of one specific prebiotic compound on the levels of several periodontopathogens

was already demonstrated however the effect of this prebiotic compound needs to be tested on cariogenic bacteria [48].

Further, some dairy food products have health benefits and have been investigated in order to use them properly [48]. It is already known that body's microbiota can be controlled and modified according to use of pre and probiotics, but an evidence of probiotic therapy on oral diseases is required [46], [48].

## CHAPTER 2

### Development of a new qPCR for *Streptococcus salivarius*



## 2.1. Introduction

Quantification of oral *streptococci* is normally made by microbial culturing, which is laborious and time-consuming and has several disadvantages [53]. However, a quantitative analysis is always required to detect, count and control bacteria associated to dental caries [39]. So, several methods have been developed to quantify and identify different bacteria in the mouth such as biochemical, immunological and genetic tests [32], [39], [54]. The majority of these methods are, however qualitative and based on bacterial detection systems that are time-consuming, laborious and with possibility of contamination [39], [54]. Therefore, the use of molecular methods such as DNA-based methodologies would solve these problems and their use have been increasing [55].

Quantitative PCR (qPCR) assay is a method based on 5'-3' exonuclease activity of *Taq* polymerase and DNA copy number [39]. qPCR enumerates the accumulation of reporter fluorescence as a result of the cleavage of the probe during PCR amplification [9], [39]. The probe consists of a specific sequence labeled with a fluorescent reporter dye and a quencher emitting fluorescence. The quencher dye avoids the extension of the probe by the polymerase and when it is cleaved it allows the accumulation of the reporter fluorescence [9]. The probe is normally marked with intercalating dyes (non-specific sequences of fluorescents dyes emitting a large fluorescence when intercalate into double-stranded DNA) [56].

This method requires only a small volume of sample and amplifies and quantifies the nucleic acid sequences at the same time and so there is no need to run a gel to see the product. Thus, qPCR is an accurate, sensitive, precise, specific, fast, reliable, powerful and useful method with a low possibility of contamination [9], [39], [56], [57].

qPCR assays are already developed for *Streptococcus mutans* and *Streptococcus sobrinus*, the main cariogenic bacteria [39]. However, the quantification of *Streptococcus salivarius* by a qPCR assay is also required due to the great interest in this microorganism as a probiotic strain. Nowadays, the interest on probiotics is growing due to their beneficial health effects [58]. *S. salivarius* is an important microorganism of the oral microbiota and it is the most abundant species of streptococcal present in the oral cavity and an early colonizer of oral surfaces with potential for use as an oral probiotic [59], [60].

*Streptococcus* species have a specific conserved locus on the dextranase gene. Dextranase gene is an enzyme that hydrolyses glucans in the plaque matrix and it could even be one of the responsible for the virulence of these strains [54].



So, in this stage a qPCR assay was developed for the culture-independent enumeration of *S. salivarius* and to maximize the specificity of the qPCR assay in environmental samples.

## 2.2. Materials and Methods

### 2.2.1. Bacterial strains and culturing conditions

In the present work *Streptococcus mutans* ATCC 25175, *Streptococcus sobrinus* ATCC 33478, *Streptococcus salivarius* TOVE-R, *Streptococcus salivarius* clinical strain, *Streptococcus salivarius* K12 and *Streptococcus salivarius* ATCC 7073 were the bacteria used. Bacteria were maintained on blood agar plates (Blood Agar Base II, Oxoid, Basingstoke, UK) supplemented with 5% sterile horse blood (Biotrading, Keerbergen, Belgium), 5 µg/ml hemin (Sigma Chemical Co, St. Louis, MO) and 1 µg/ml menadion. One day before each experiment, bacteria were collected from blood agar plates and incubated overnight in 10 ml Brain-Heart Infusion (BHI) broth (Becton, Dickinson and Company, France) at 37 °C in a 5% CO<sub>2</sub> environment. Bacterial concentration was adjusted by optical density measurements at a wavelength of 600 nm (Smartspec 3000, BioRad, USA).

### 2.2.2. Design of qPCR primers and probe

The forward primer (Ssal442F) was based on the dextranase gene from *S. salivarius* and was selected from Igarashi et al. (2001). The reverse primer (Ssal615R) and probe (Ssal497T) were also based on the dextranase gene from *S. salivarius* and were designed with primer 3 software. A search with BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and Probe Match [61] was carried out in order to assess, *in silico*, the homology of the selected primers and the probe with unrelated sequences. The primers-probe set for primer-dimers, melting temperature, hairpin configuration and GC content were checked with OligoAnalyzer (Integrated DNA Technologies, Coralville, IA, USA). Primers and probe were synthesized by Eurogentec (Seraing, Belgium). The probe was 5' labeled with a fluorescent dye as a reporter, FAM (6-carboxyfluorescein), and another fluorescent dye as a quencher, 3' TAMRA (6-carboxytetramethylrhodamine). A PCR assay against *S. mutans*, *S. sobrinus* and 4 different strains of *S. salivarius* was performed as a confirmation of the forward and reverse primer. The PCR steps consisted of an initial 1 min at 94 °C, followed by 26 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, and an additional cycle of 5 min at 72 °C. PCR fragments were checked in an electrophoresis on 1% agarose gel.

### **2.2.3. Construction of the qPCR plasmid standard**

A fragment of 192 bp of the *S. salivarius* dextranase gene was used as a standard for the qPCR and was amplified with the forward primer Ssal442F (5' - AACGTTGACCTTACGCTAGC -3') and lately designed reverse primer Ssal615R (5' - ACCGTAACGTGGGAAACTG -3'). This fragment was purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany), cloned with the pGEM-T easy plasmid vector system (Promega, Madison, WI, USA) and used to transform *Escherichia coli* DH5 $\alpha$ . High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to isolate the plasmids from the clones, according to the manufacturer's instructions. The validation of the DNA sequence of the plasmid was made by sequencing with the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Dye ex sequencing purification kit (Qiagen) was used to purify the sequencing product and ABI 310 Genetic Analyzer (Applied Biosystems) was used to analyze it. Plasmid concentration and purity were determined with GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech). Each qPCR run was performed with a 10-fold dilution series of the plasmid to construct the standard curve.

### **2.2.4. qPCR optimization**

A quantitative Polymerase Chain Reaction (qPCR) assay was performed with a CFX96 Real-Time System (BioRad, CA, USA). Taqman 5' nuclease assay PCR method was used in order to detect and quantify bacterial DNA. Taqman reactions contained 12.5  $\mu$ l mastermix (Eurogentec, Seraing, Belgium), 4.5  $\mu$ l sterile water, 1  $\mu$ l of each primer and probe, and 5  $\mu$ l template DNA. Individual primer concentrations ranging from 100 to 900 nM and probe concentration ranging from 50 to 200 nM were tested. Assay conditions steps involved an initial step for 2 min at 50 °C, followed by a denaturation step at 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

### **2.2.5. Comparison between qPCR and microbial culturing**

An overnight culture of *Streptococcus salivarius* TOVE-R was used to compare qPCR and microbial culturing. For microbial culturing, 10-fold serial dilutions in sterile saline were used. For that, 50  $\mu$ l of each dilution was plated on blood agar plates with a spiral platter (L.E.D. Techno). The plates were incubated in a 37 °C and 5% CO<sub>2</sub> environment for 3 days and the colony-forming units (CFU) were determined considering plates with colony counts between 20 and 300. For qPCR, 100  $\mu$ l aliquots of each dilution were used for DNA extraction with the QIAamp DNA mini kit according to the manufacturer's instructions (QIAGEN). qPCR was

performed as described previously. The experiment was repeated five times and values were log-transformed to calculate an orthogonal regression.

## 2.3. Results and Discussion

### 2.3.1. Design of qPCR primers and probe

qPCR assay for *S. salivarius* could not be designed against 16S rRNA gene such as for other oral bacteria because this is not specific for every species of *Streptococcus*. So, a conserved locus of the dextranase gene of *S. salivarius* ranging from base number 442 to base number 615 was the base of the design of qPCR primers due to its specific and conserved sequences [54]. The design of the PCR primers was developed on the basis of the comparison of the nucleotide sequences of the dextranase gene of *S. salivarius*, *S. mutans* and *S. sobrinus* and the consensus sequence of the alignment served as template (Table 1).

**Table 1 – Comparison of the sequences of primers and probe designed for different strains of *mutans streptococci***

Strain	Accession number	Primer Ssal442F	Primer Ssal497T	Primer Ssal615R
57.I	CP002888.1	AACGTTGACCTTACGCTAGC	GTAGCGTCAGAGTGGTTGAC	CAGTTTTCCACGTTACGGT
JIM8777	FR873482.1	AACGTTGACCTTACGCTAGC	GTAGCGTCAGAGTGGTTGAC	CAGTTTTCCACGTTACGGT
CCHSS3	FR873481.1	AACGTTGACCTTACGCTAGC	GTAGCGTCAGAGTGGTTGAC	CAGTTTTCCACGTTACGGT

Primers for PCR were obtained by comparison of the nucleotide sequences of the dextranase genes of *S. salivarius*, *S. mutans* and *S. sobrinus*. The forward primer (Ssal442F) was kept from a PCR assay from Igarishi et al. (2001) and a new reverse primer (Ssal615R) was designed reducing the fragment length from 2271 base pairs (bp) to 192 bp. A qPCR probe (Ssal497T) was designed between the beginning of the forward primer and the end of the reverse primer (Table 2).

**Table 2 – qPCR primers and probe: Guanine-Cytosine content (% GC), Melting temperature (Tm)**

Oligonucleotide name	Sequence (5'-3')	Position	%GC	Tm (°C)
Forward primer: Ssal442F	AACGTTGACCTTACGCTAGC	442 to 458	50	60
Reverse primer: Ssal615R	ACCGTAACGTGGGAAACTG	615 to 634	45	58
Taqman probe: Ssal497T	GTAGCGTCAGAGTGGTTGAC	497 to 516	55	62

A PCR assay against *S. mutans*, *S. sobrinus* and four strains of *S. salivarius* was carried out to test the specificity of the primers (Figure 5).

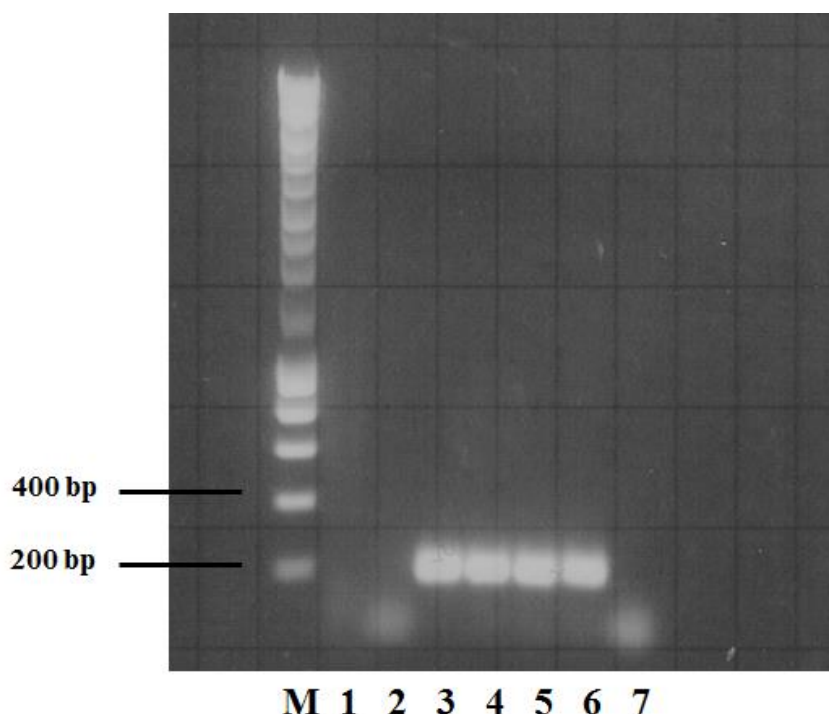


Figure 5 - PCR amplification for detection of the dextranase gene of oral streptococcal species by a PCR and a DNA probe (Ssal497T) with Ssal442F and Ssal615R primer pair: *S. mutans* (lane 1), *S. sobrinus* (lane 2), *S. salivarius* ATCC 7073 (lane 3), *S. salivarius* K12 (lane 4), *S. salivarius* clinical strain (lane 5), *S. salivarius* TOVE-R (lane 6). Negative control (lane 7) was made with physiological water.

A band at 200 bp was displayed for all strains of *S. salivarius* (lanes 3, 4, 5 and 6) and no amplification was observed for *S. mutans* (lane 1) and for *S. sobrinus* (lane 2). Looking at negative control, made with physiological water (lane 7), no amplification can be observed meaning no detection of contamination in this PCR assay. So, PCR amplification was verified only for *S. salivarius* strains. This indicates that the PCR primers and probe were specific for this species.

### 2.3.2. qPCR optimization

Data were collected during the annealing phase of qPCR assay. The optimal qPCR primers concentration was determined by titration assays. The best concentrations were obtained with 400 nM for the forward primer and 100 nM for the reverse primer. For the probe, the best result was obtained for a concentration of 100 nM. The reaction efficiency was on average 94.87% ( $\pm 2.36$  SD). The reaction efficiency was calculated from the slope of the standard curve  $[= 10^{(-1/\text{slope})} - 1]$ . The lowest reproducible detection level of the qPCR was 4 plasmids per reaction, each containing one target sequence.

### 2.3.3. Comparison between qPCR and microbial culturing

Microbial culturing of *S. salivarius* was carried out and compared with qPCR for the same strain (Figure 6).

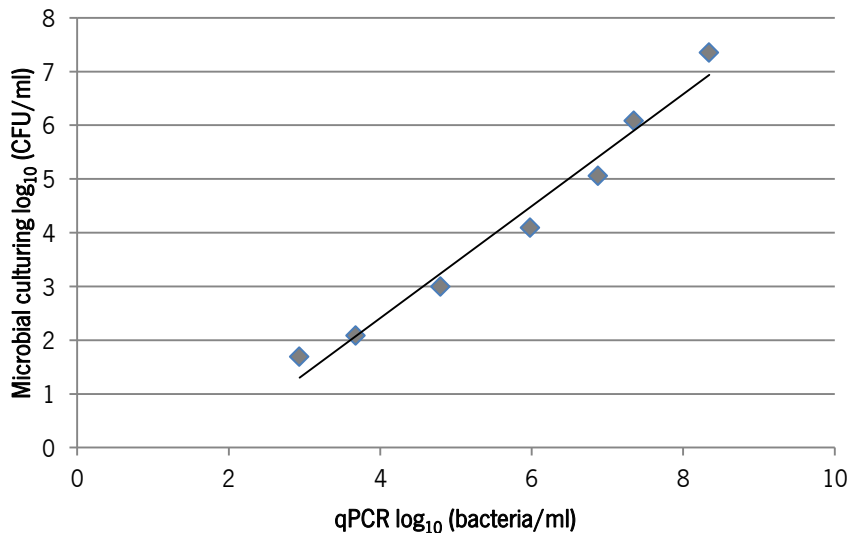


Figure 6: Number of colony forming units (CFU) of *Streptococcus salivarius* (Y-axis) versus quantification of *Streptococcus salivarius* by quantitative Polymerase Chain Reaction (qPCR) (X-axis). Correlation coefficient:  $R^2=0.9741$ .

Comparing these two different culture techniques for *S. salivarius*, a high degree of correlation ( $R^2 = 0.9741$ ) can be observed. This means a clear linear relationship between the results obtained through both techniques. Further, qPCR values are almost 1.47 times more bacteria than microbial culturing, which could be explained by counting of live and dead cells by this molecular method [55]. So, it was concluded that qPCR assay for *S. salivarius* allows the quantification of this strain with a linear relationship to results obtained by microbial culturing.

## 2.4. Conclusion

A qPCR assay for the enumeration of *S. salivarius* was needed. Primers were chosen based on the dextranase gene of *S. salivarius*, a conserved region and tested on 4 different strains of *S. salivarius* and 2 other strains (*S. mutans* and *S. sobrinus*). Only *S. salivarius* were amplified, whereby it can be concluded that this qPCR assay is highly specific for *S. salivarius* and allowing the detection and identification of this bacterium on environmental samples. Further, qPCR were compared with microbial culturing and results presented a linear relationship corroborating the correct quantification of this strain by qPCR.

In conclusion, a TaqMan qPCR assay specific for *S. salivarius* was developed based on the dextranase gene.

## CHAPTER 3

### Effect of a prebiotic compound on cariogenic and saliva bacteria



### 3.1. Introduction

The mouth is a complex ecosystem with several microorganisms creating dynamic interactions and growing as a biofilm, called dental plaque. However, under certain conditions, changes in the dental plaque and in the oral environment can lead to serious infections, such as periodontitis and dental caries [62].

According to the 'ecological plaque hypothesis' in dental caries, when an increase in the frequency of fermentable carbohydrates consumption is verified, pH decreases and acid-tolerating bacteria proliferate increasing cariogenic bacteria in dental plaque [7], [10].

Nowadays traditional therapy (mechanical instrumentation and antimicrobial treatment), as well as alternative ones, as vaccination are not the most appropriate since they are very invasive and can cause resistance [20], [46]. So, alternative ways are required, and probiotics and prebiotics are emerging in diverse fields like a viable hypothesis in the prevention and treatment of several diseases [21], [47], [52].

Probiotics are live microorganisms which, when administered in correct quantities, confer benefits to the host [21]. *Lactobacilli* and *bifidobacteria* are the most common probiotic strains [62]. Some *lactobacilli* strains with probiotic properties can even be found in the mouth. For examples, some studies have already demonstrated the potential of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* GG in *Streptococcus mutans* reduction through yoghurt consumption [62]. Other properties of probiotics are cancer risk reduction, immune response induction and antimicrobial potential [62]. *Streptococcus salivarius* is an oral probiotic strain that lives in the oral cavity of healthy people. For example, it was already proven that *S. salivarius* can reduce volatile sulphur compounds [62]. These compounds are derived from the bacterial degradation of sulphur containing amino acids in the oropharynx and are involved in halitosis (bad breath) [62].

Prebiotics can be a complement to probiotics in the treatment and control of oral diseases stimulating the growth of beneficial indigenous bacteria. Prebiotics are able to promote the growth and activity of beneficial microorganisms and to inhibit the growth and activity of pathogenic microorganisms. So, the combination of pre and probiotics can improve oral health conditions [62].

In the case of the oral cavity, oral probiotics need to be able to adhere and colonize oral surfaces and to be part of the biofilm in order to stimulate the reduction of cariogenic bacteria levels [62]. So, the identification of oral strains is a very important step. However, cultivation methods cannot detect unculturable strains. Unculturable strains are live bacteria with metabolic



activity but unable to develop into colonies on culture media as a result of natural stress [63]. So, culture-independent molecular methods are required such as quantitative Polymerase Chain Reaction (qPCR) or Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis (PCR-DGGE).

qPCR is an accurate and precise method that enumerates the PCR product during amplification [56]. PCR-DGGE is a cultivation-independent molecular fingerprinting technique that does not require cultivation, and that can be used for the identification of cultivable and unculturable microorganisms in the oral cavity [64]. The separation is based on electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels [65].

In this chapter, a prebiotic compound (C7) was assessed in order to see its effect in the growth of two main cariogenic bacteria (*S. mutans* and *S. sobrinus*) and one probiotic strain (*S. salivarius*). The aim was to discover if the addition of this compound leads to a decrease in the concentration of these cariogenic bacteria. Further, the effect of this compound on the microorganisms present in the human saliva was also tested to verify any microbial shift in the presence of a probiotic strain (*S. salivarius*).

## 3.2. Materials and Methods

### 3.2.1. Bacterial strains and culturing conditions

*Streptococcus sobrinus* ATCC 33478 and *Streptococcus mutans* ATCC 25175 were the cariogenic bacteria used. *Streptococcus salivarius* K12 was used as a beneficial bacterium. All bacteria were maintained on blood agar plates (Blood Agar Base II, Oxoid, Basingstoke, UK) supplemented with 5% sterile horse blood (Biotrading, Keerbergen, Belgium), 5 µg/ml hemin (Sigma Chemical Co, St. Louis, MO) and 1 µg/ml menadion. Bacteria were collected from blood agar plates one day before each experiment and incubated overnight in 10 ml Brain-Heart Infusion (BHI) broth (Becton, Dickinson and Company, France) at 37 °C in a 5% CO<sub>2</sub> environment.

Different media were used in order to find a selective substratum for *S. salivarius*, *S. mutans* and *S. sobrinus*: Mitis-Salivarius (MS) agar (Becton, Dickinson and Company, France), Trypticase Yeast Cysteine Sucrose Bacitracin (TYCSB) agar (Becton, Dickinson and Company, France) and blood agar. MS agar and TYCSB agar were tested as a possible culture medium and blood agar was used as a positive control.

### 3.2.2. Media testing

To find an appropriate medium for specific growth of *S. mutans* and *S. sobrinus*, cultures of each bacteria and *S. salivarius* were grown overnight and the concentration was adjusted to  $1 \times 10^7$  cells/ml by measuring the optical density (OD) at a wavelength of 600 nm (Smartspec 3000, BioRad, USA). 100  $\mu$ l of the bacterial suspension containing  $1 \times 10^7$  bacteria was spread with a sterile loop on both MS and TYCSB plates and were incubated at 37 °C in a 5% CO<sub>2</sub> environment for 3 days. Bacterial growth was observed by light microscopy.

### 3.2.3. Growth in dual-species model

Overnight cultures of cariogenic bacterium (*S. mutans* or *S. sobrinus*) and *S. salivarius* in BHI were used. The optical density was measured at 600 nm and suspensions were prepared containing approximately  $1 \times 10^7$  cells/ml. Experiments were carried out in a dual-species model in 24 wells plate with 1 ml of the cariogenic bacteria (*S. mutans* or *S. sobrinus*) and 1 ml of *S. salivarius*. Different conditions were tested: the cariogenic bacterium with or without C7 compound (200  $\mu$ l) and both species (the cariogenic bacterium with *S. salivarius*) with or without C7 compound (200  $\mu$ l). The positive control was made with glucose (200  $\mu$ l) and was tested not only with the cariogenic bacterium alone, but also with both species. The negative control was made only with BHI. *S. salivarius* was replaced by BHI in the conditions where only the cariogenic strain was tested in order to get the same volume. A final concentration of 1 mg/ml was used for C7 compound and for glucose. The different components of each condition assessed can be observed in Table 3.

**Table 3: Conditions used in dual-species model growth with each one of cariogenic bacteria (*Streptococcus mutans* or *Streptococcus sobrinus*). Prebiotic compound (C7)**

Condition			
	Main bacteria	Second bacteria	Added sugar
A	Cariogenic bacterium	–	–
B	Cariogenic bacterium	–	Glucose
C	Cariogenic bacterium	<i>S. salivarius</i>	Glucose
D	Cariogenic bacterium	–	C7
E	Cariogenic bacterium	<i>S. salivarius</i>	C7
F	Cariogenic bacterium	<i>S. salivarius</i>	–

At 0 h, 100  $\mu$ l of each condition were used to make dilutions of each condition, whereas 500  $\mu$ l of each sample was kept at -20 °C until DNA extraction was performed. 50  $\mu$ l of each dilution was plated on TYCSB plates with a spiral platter (L.E.D. Techno). The plates were incubated in a 37 °C and 5% CO<sub>2</sub> environment for 3 days and the colony-forming units (CFU) were determined in plates with colony counts between 20 and 300. Selective microbial plating for

the cariogenic strain tested was carried out also at 24 h and 48 h. Furthermore, at 24 h and 48 h, 300 µl of each condition was used to measure the pH. This experiment was repeated at least three times.

### **3.2.4. DNA extraction and quantitative Polymerase Chain Reaction (qPCR)**

The samples were kept at -20 °C until use. The DNA extraction was performed with the QIAamp DNA mini kit according the manufacturer's instructions (Qiagen). A quantitative polymerase chain reaction (qPCR) assay was performed with a CFX96 Real-Time System (BioRad, CA, USA). In order to quantify the concentration of bacterial DNA of *S. mutans* and *S. salivarius* present in the samples Taqman 5' nuclease assay PCR method was used. The construction of primers and probe for *S. mutans* was based upon the glucosyltransferase B (*gtfB*) gene and the primers used can be observed in Table 4. For *S. salivarius* primers and probe were designed based on the dextranase gene of *S. salivarius* JCM5707 (Table 4). And for *S. sobrinus* primers and probe (Table 4) were constructed based upon the glucosyltransferase T (*gtfT*) gene.

**Table 4: Sequence and final concentrations of primers and probe used in quantitative Polymerase Chain reaction (qPCR) assay for *Streptococcus salivarius*, *Streptococcus mutans* and *Streptococcus sobrinus***

Primers and probe	Sequence (5'-3')	Concentration (nM)
<i>S. salivarius</i> forward	5'-AACGTTGACCTTACGCTAGC-3'	400
<i>S. salivarius</i> reverse	5'-ACCGTAACGTGGGAAACTG-3'	100
<i>S. salivarius</i> probe	5'-GTAGCGTCAGAGTGGTTGAC-3'	100
<i>S. mutans</i> forward	5'-GCCTACAGCTCAGAGATGCTATTCT-3'	900
<i>S. mutans</i> reverse	5'-GCCATACACCACTCATGAATTGA-3'	900
<i>S. mutans</i> probe	5'-TGGAAATGACGGTCGCCGTTATGAA-3'	900
<i>S. sobrinus</i> forward	5'-TTCAAAGCCAAGACCAAGCTAGT-3'	200
<i>S. sobrinus</i> reverse	5'-CCAGCCTGAGATTCAGCTTGT-3'	200
<i>S. sobrinus</i> probe	5'-CCTGCTCCAGCGACAAAGGCAGC-3'	250

Taqman reactions contained 12.5 µl mastermix (Eurogentec, Seraing, Belgium), 4.5 µl sterile water and 1 µl of each primer and probe. For *S. mutans* qPCR steps consisted of an initial 2 min at 50 °C, followed by a denaturation step for 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. For *S. salivarius* qPCR steps involved an initial step for 2 min at 50 °C, followed by a denaturation step at 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. qPCR steps used for *S. sobrinus* consisted of an initial step at 50 °C for 2 min, followed by 10 min at 95 °C and 60 cycles of 95 °C for 15 s and 58 °C for 1 min. A plasmid standard curve was used in order to quantify the concentration of bacterial DNA of *S. mutans* and *S. salivarius*.

### **3.2.5. Saliva collection and preparation**

Saliva was collected from 4 healthy volunteers in a sterile tube and 9 ml saliva was pooled. *S. salivarius* K12 was washed, after overnight growth, the culture was centrifuged at 7000 rpm for 10 min and the pellet was re-suspended in physiological water. The concentration was adjusted to  $1 \times 10^9$  cells/ml by optical density (OD) measured at 600 nm (Smartspec 3000, BioRad, USA). A suspension of 900  $\mu$ l of *S. salivarius* was added to the pooled saliva and it was mixed and divided in 2 parts. One part was centrifuged at 7500 rpm for 10 min and the pellet was re-suspended in BHI. C7 compound (1 mg/ml, final concentration) was added to each part (with and without centrifugation) and physiological water was used as negative control. Each condition was divided and then one part was incubated in an aerobic incubator at 37 °C and another part was incubated in an anaerobic incubator (80% N<sub>2</sub>, 10% H<sub>2</sub> and 10% CO<sub>2</sub>). At 0, 24 and 48 h, 100  $\mu$ l of the suspension obtained in each condition was stored at -20 °C until DNA extraction was performed.

### **3.2.6. DNA extraction, PCR-DGGE assay**

The DNA was extracted using QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen). Extracted DNA was amplified by PCR assay using universal bacterial 16S rDNA primers: P338F (forward), 5' – CAGGCCTAACACATGCAAGTC – 3'; P518R (reverse), 5' – ATTACCGCGGCTGCTGG – 3'. Reactions contained 1  $\mu$ l of each primer, 0.5  $\mu$ l of AmpliTaq® DNA polymerase (Roche), 1.2  $\mu$ l of MgCl<sub>2</sub>, 1.6  $\mu$ l of deoxynucleoside triphosphate (dNTP), 10.7  $\mu$ l of sterile Milli-Q water, 2  $\mu$ l of 10X Taq Buffer with KCL (MgCl<sub>2</sub> free) (Fermentas), 0.625  $\mu$ l of bovine serum albumin and 2  $\mu$ l of each sample. The PCR steps consisted of an initial 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 53 °C for 1 min and 72 °C for 1 min and an additional cycle of 12 min at 72 °C. *S. salivarius* was used as positive control. Quality of PCR product was evaluated by electrophoresis in 1% agarose gel run at 90 V for 20 min.

Denaturing Gradient Gel Electrophoresis (DGGE) was performed using the DCode™ Universal Mutation Detection System (BioRad, Hercules, California, USA) based on the protocol of Muyzer et al. (1993). So, 8% (w/v) of polyacrylamide gels in 1X Tris-acetate-ethylenediaminetetraacetic (TAE) diluted from 50X TAE buffer stock (121.24 g Tris, 20.5 g sodium acetate anhydrate, 9.8 g ethylenediaminetetraacetic (EDTA), 250 ml Milli-Q water and pH adjusted to 7.8 to a final volume of 500 ml) were made with denaturing gradient ranging from 45 to 60% in order to load PCR products. Denaturing solutions were made from a 0% denaturation buffer [10 ml 40% acrylamide/Bis Solution (BioRad), 2.5 ml 2% Bis Solution (BioRad), 1 ml 50X

TAE and Milli-Q water till 50 ml] and 60% denaturation buffer [10 ml 40% acrylamide/Bis Solution (BioRad), 2.5 ml 2% Bis Solution (BioRad), 12.5 g urea (Sigma), 12 ml formamide (Sigma), 1 ml 50X TAE and Milli-Q water till 50 ml]. Polyacrylamide gels were polymerized by adding ammonium persulfate solution 10% (BioRad, Hercules, California, USA) and TEMED into each of the denaturing solutions right before pouring the gradient gel. Electrophoresis was performed at a constant voltage of 75 V at 60 °C for 16 h in 1X TAE buffer. DGGE standard markers were used and PCR products were directly loaded in each lane. *S. salivarius* was used as positive control. Gels were rinsed and stained in 200 ml 1X TAE buffer with 13 µl Gel Red Nucleic Acid Stain (Biotium) for 30 min. The DGGE images were digitally captured and recorded with UV transillumination. The processing of the DGGE gels was made with the Bionumerics software 2.0 (Applied Maths, Kortrijk, Belgium).

### 3.3. Results

#### 3.3.1. Selective medium for *Streptococcus salivarius*, *Streptococcus mutans* and *Streptococcus sobrinus*

To test the effect of a prebiotic compound on cariogenic bacteria, a selective medium for cariogenic strains (*S. mutans* and *S. sobrinus*) had to be defined, as well as, for the beneficial strain, *S. salivarius*. Different media were used and each strain was plated on each different medium.

After CO<sub>2</sub> incubation for 3 days, growth was observed on MS agar plates with every strain used: *S. salivarius* (Figure 7-IA), *S. mutans* (Figure 7-IB) and *S. sobrinus* (Figure 7-IC). On the other hand, on TYCSB plates growth was only observed for *S. mutans* (Figure 7-IIB) and for *S. sobrinus* (Figure 7-IIC) and non-growth was seen for *S. salivarius* (Figure 7-IIA).

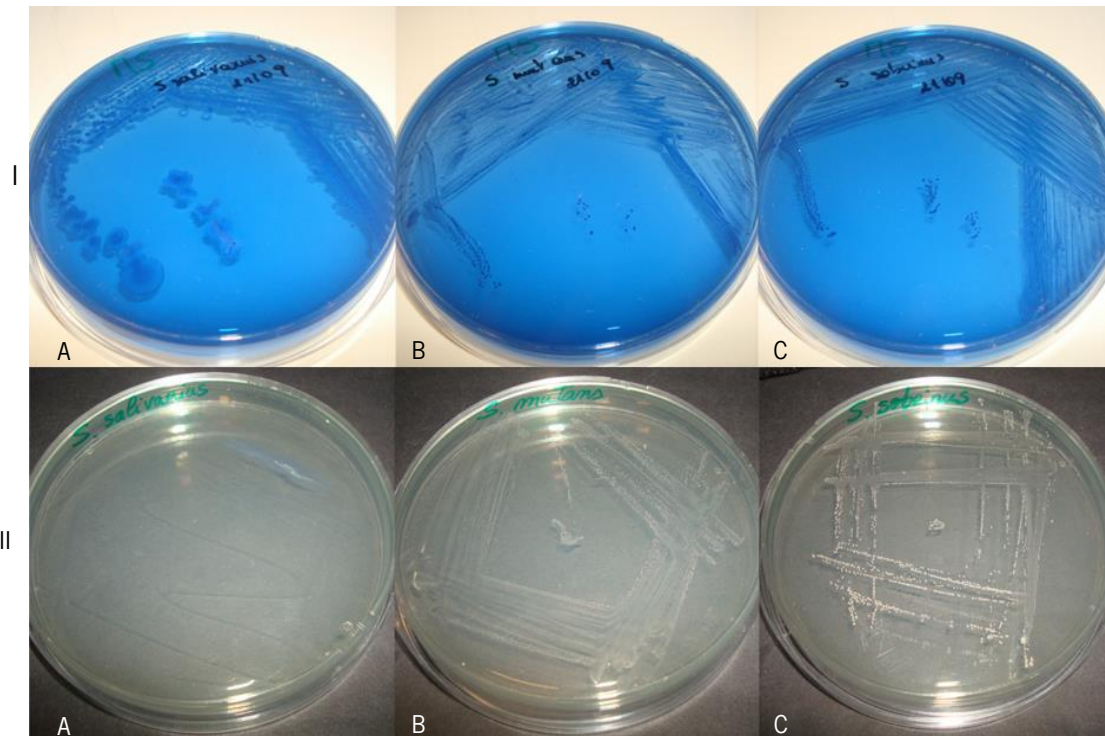
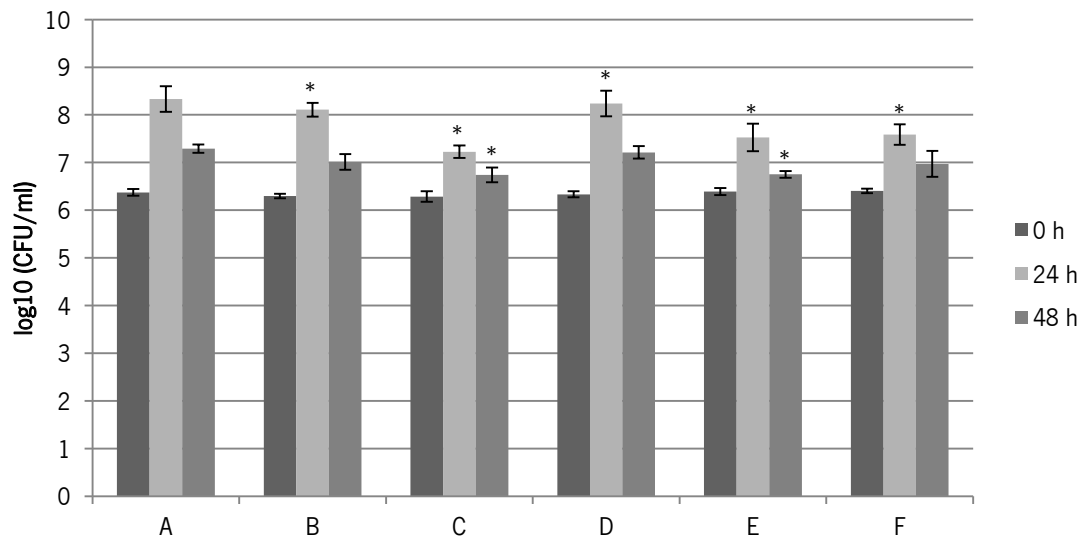


Figure 7: Growth on Mitis-Salivarius agar plates (I) and on Trypticase Yeast Cysteine Sucrose Bacitracin (TYCSB) plates (II). (A): *Streptococcus salivarius*, (B): *Streptococcus mutans* and (C): *Streptococcus sobrinus*.

It was concluded that MS medium could not be used as a selective medium for any strain, but otherwise TYCSB medium could be used for *S. mutans* and for *S. sobrinus*. A selective medium for *S. salivarius* was not found.

### 3.3.2. Effect of a prebiotic compound on cariogenic bacteria

The effect of the prebiotic compound (C7) on *S. mutans* in dual species model with *S. salivarius* was tested first (Figure 8).



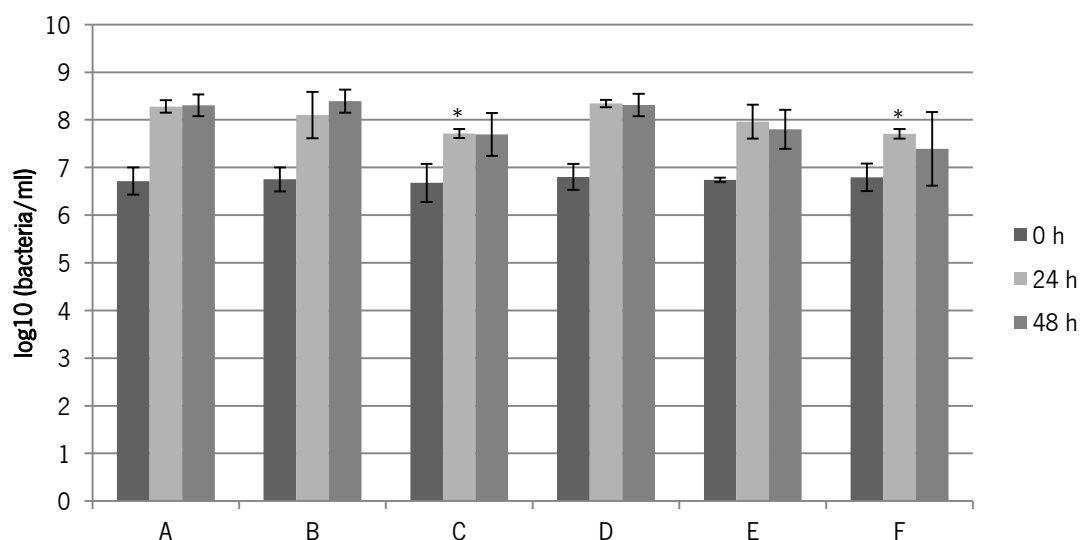
**Figure 8: Number of colony forming units (CFU) of *Streptococcus mutans* in dual species model at 0 h, 24 h and 48 h:** Growth of *S. mutans* testing the effect of C7 compound with (E) or without (D) the presence of *S. salivarius* and glucose with (C) or without (B) *S. salivarius*. The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and *S. salivarius* without addition of glucose (F). Standard errors of the mean (n=3) are represented by error bars. Significance ( $p < 0.05$ ) between the control (only *S. mutans*) and test series was determined using Student's *t*-test and are marked with \*.

An increase was detected in *S. mutans* concentration in all conditions after 24 h, when compared to 0 h. At 24 h, significant differences were detected in all conditions ( $p < 0.05$ ), when compared to the control (condition A). However, looking at Figure 8, *S. mutans* had less growth in all conditions where *S. salivarius* is present. The lowest value can be observed when *S. mutans* is conjugated with *S. salivarius* and glucose (condition C).

At 24 h, another significant difference ( $p < 0.05$ ) could be found when *S. mutans* was conjugated with *S. salivarius* (condition F) in comparison to the presence of glucose (condition C).

A reduction in all conditions was detected from 24 h to 48 h. A significant reduction could be observed when *S. mutans* is present together with *S. salivarius* and glucose (condition C) and C7 (condition E) ( $p < 0.05$ ), when compared with only the cariogenic bacterium (condition A).

Quantitative Polymerase Chain Reaction (qPCR) analysis was also carried out to see the effect of a prebiotic compound on *S. mutans* in dual species model, under the presence of the two compounds, C7 and glucose, and the results can be seen in Figure 9.

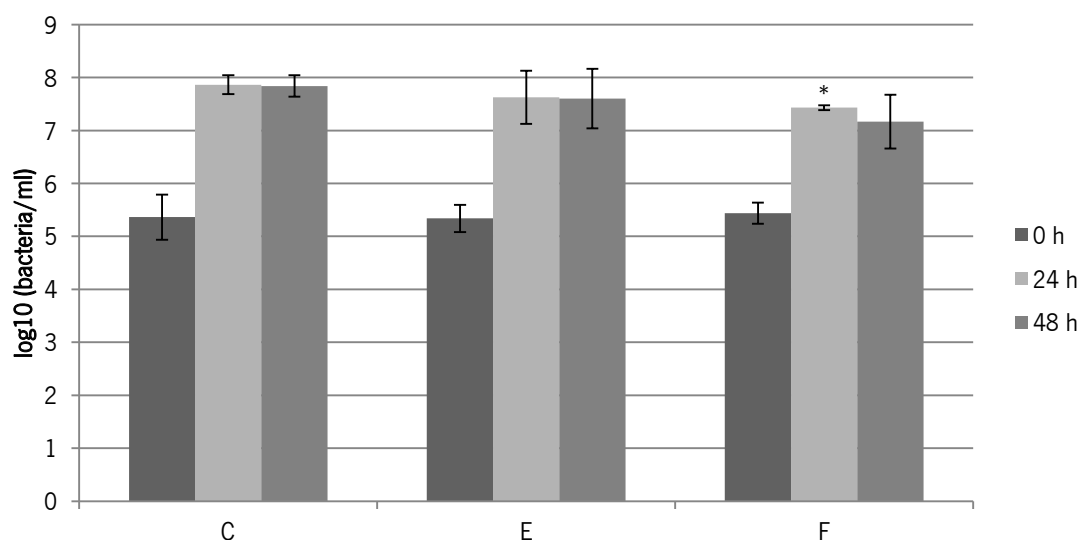


**Figure 9: Number of *Streptococcus mutans* in dual species model at 0 h, 24 h and 48 h determined by quantitative Polymerase Chain Reaction (qPCR).** The effect on *S. mutans* of C7 compound with (E) or without (D) the presence of *S. salivarius* and glucose with (C) or without (B) *S. salivarius*. The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and *S. salivarius* without addition of glucose (F). Standard errors of the mean (n=2 for 0 h and n=3 for 24 h and for 48 h) are represented by error bars. Statistically significant differences (p<0.05) between the control (only *S. mutans*) and test series were determined using Student's *t*-test and are marked with \*.

From Figure 9, it is possible to observe that after 24 h there was less growth of *S. mutans* when *S. salivarius* is present (condition F) and when these bacteria are together with glucose (condition C). These reductions are significant (p<0.05) in comparison with control (condition A). After 48 h no significant reductions were detected.

The use of qPCR allowed also the determination of the number of *S. salivarius* present in each condition (Figure 10).





**Figure 10: Number of *Streptococcus salivarius* in *Streptococcus mutans* dual species model experiment at 0 h, 24 h and 48 h determined by quantitative Polymerase Chain Reaction (qPCR):** *S. mutans* conjugated with *S. salivarius* and glucose (C), *S. mutans* together with *S. salivarius* and the C7 compound (E). The control was made with the cariogenic bacterium and *S. salivarius* (F). Standard errors of the mean (n=3) are represented by error bars. Statistically significant differences (p<0.05) between the cariogenic bacterium (*S. mutans*) together with the probiotic bacterium (*S. salivarius*) with and without glucose are marked with \* and was determined using Student's t-test.

A significant increase (p<0.05) was detected when *S. salivarius* is present together with *S. mutans* (condition F) after 24 h, when compared with the presence of these bacteria together with glucose (condition C). A small increase was verified when C7 compound or glucose are present, comparing with condition F, but not significant. After 48 h, no significant differences were detected.

In order to complement the study, since oral bacteria are able to affect oral environment, the pH was measured for all conditions at both 24 h and 48 h (Table 5).

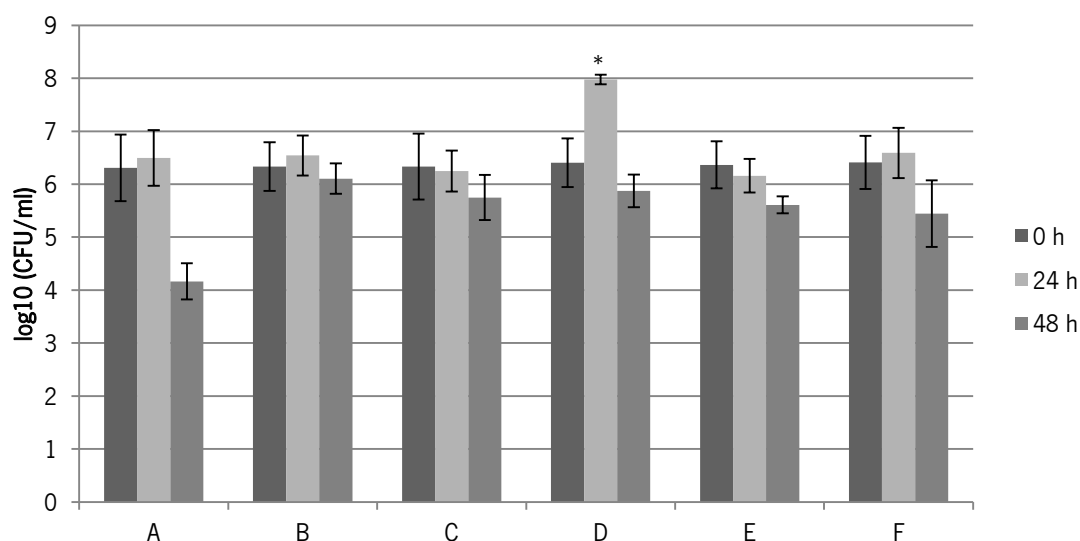
**Table 5: pH values for *Streptococcus mutans* in dual species model at 24 h and 48 h:** Assessment of pH variation of *S. mutans* culture testing the effect of C7 compound with (E) or without (D) the presence of *S. salivarius* and glucose with (C) or without (B) *S. salivarius*. The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and *S. salivarius* without addition of glucose (F)

Condition	pH values					
	A	B	C	D	E	F
24 h	5.57	4.93	4.85	5.55	4.84	5.41
48 h	5.62	4.99	4.86	5.57	4.85	5.41

An average pH of 5.57 was determined after 24 h of the start of the experiment in the control, only with *S. mutans* (condition A). A lower pH value was detected for almost all the conditions. There is a slight difference between *S. mutans* and glucose (condition B) and *S. mutans* and glucose with *S. salivarius* (condition C) and *S. mutans* together with *S. salivarius* and C7 (condition E). Besides these slight differences, the pH in condition B is always slightly higher.

An extra pH decrease was seen when *S. salivarius* was present together with glucose (condition C) and the same was observed when *S. salivarius* was present together with C7 compound (condition E) when comparing with only *S. salivarius* (condition F), all of them with *S. mutans*. The values for 48 h were almost the same and no variations were detected (Table 5).

Afterwards, the effect of a prebiotic compound for *S. sobrinus*, the other cariogenic bacterium used in this study, in dual species model with *S. salivarius* was tested (Figure 11).

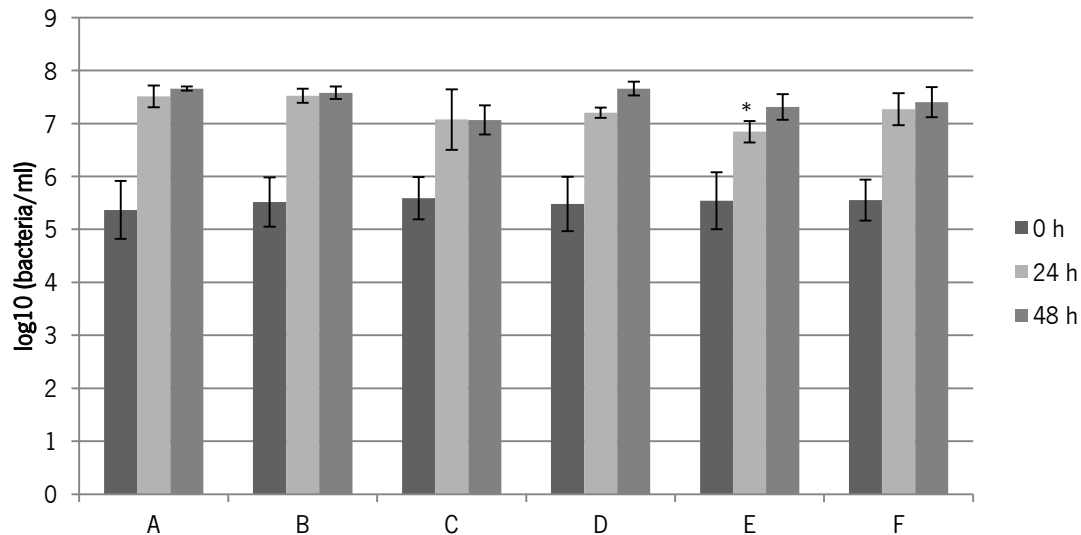


**Figure 11: Number of colony forming units (CFU) of *Streptococcus sobrinus* in dual species model at 0 h, 24 h and 48 h:** Growth of *S. sobrinus* testing the effect of C7 compound with (E) or without (D) the presence of *S. salivarius* and glucose with (C) or without (B) *S. salivarius*. The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and *S. salivarius* without addition of glucose (F). Standard errors of the mean (n=3) are represented by error bars. Significance ( $p < 0.05$ ) between the control (only *S. sobrinus*) and test series was determined using Student's *t*-test and are marked with \*.

A large and significant increase ( $p < 0.05$ ) can be observed after 24 h when *S. sobrinus* is present together with the C7 compound (condition D), if it is compared with *S. mutans* alone (condition A).

After 48 h (Figure 11) it was observed that the increase in condition D was no longer present and no more significant differences were detected. Further, the control value (condition A) seems to be very low.

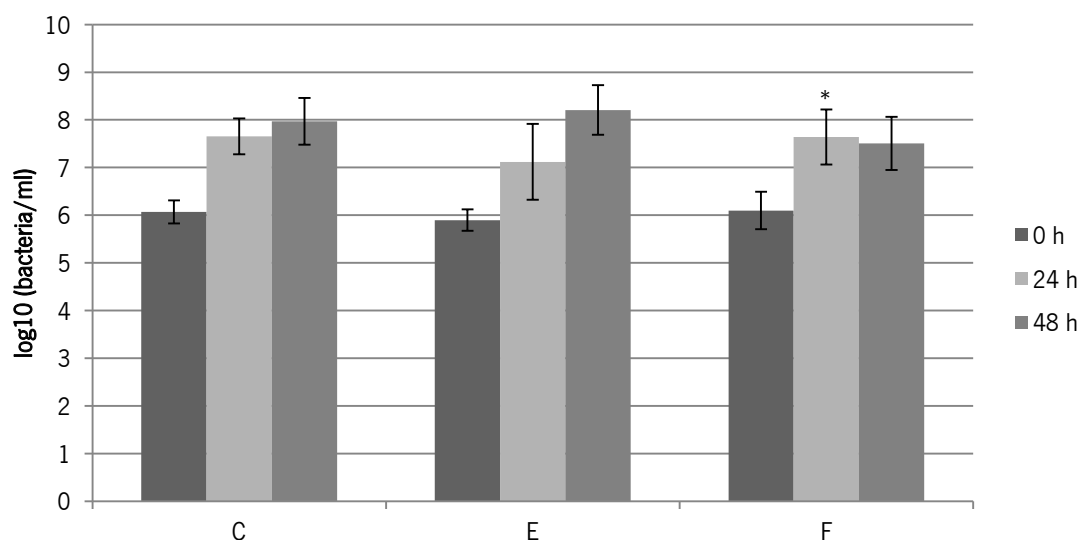
qPCR analysis was also carried out to see the effect of a prebiotic compound on *S. sobrinus* in dual species model in real time and results can be seen in Figure 12.



**Figure 12: Number of *Streptococcus sobrinus* in dual species model at 0 h, 24 h and 48 h determined by quantitative Polymerase Chain Reaction (qPCR):** *S. sobrinus* culture testing the effect of C7 compound with (E) or without (D) the presence of *S. salivarius* and glucose with (C) or without (B) *S. salivarius*. The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and *S. salivarius* without addition of glucose (F). Standard errors of the mean (n=3) are represented by error bars. Significance (p<0.05) between the control (only *S. sobrinus*) and test series was determined using Student's t-test and are marked with \*.

Looking after 24 h, conditions C, E and F show apparently a lesser growth than the others. A significant difference (p<0.05) was detected in the presence of *S. salivarius* together with *S. sobrinus* and C7 compound (condition E), when compared with control only with *S. sobrinus* (condition A). Another significant difference (p<0.05) was obtained comparing the growth of *S. sobrinus* together with *S. salivarius* with and without the C7 compound. On the other hand, the large increase seen in the culturing results on condition D (Figure 11) is no more visible.

qPCR analysis for *S. salivarius* in *S. sobrinus* dual species model experiment is displayed in Figure 13. Values at the beginning of the experiment are similar.



**Figure 13: Growth of *Streptococcus salivarius* in *Streptococcus sobrinus* dual species model experiment at 0 h, 24 h and 48 h with quantitative Polymerase Chain Reaction (qPCR):** *S. sobrinus* conjugated with *S. salivarius* and glucose (C), *S. sobrinus* together with *S. salivarius* and the C7 compound (E). The control was made with the cariogenic bacterium and *S. salivarius* (F). Standard errors of the mean (n=3) are represented by error bars. Statistically significant differences (p<0.05) between the cariogenic bacterium (*S. sobrinus*) together with the probiotic bacterium (*S. salivarius*) with and without C7 compound are marked with \* and was determined using Student's *t* test.

Growth of *S. salivarius* after 24 h was significant (p<0.05) without the addition of any carbon source (glucose or C7 compound) (condition F), when compared with addition of C7 compound (condition C). Further, the lesser growth was verified when C7 compound is present together with *S. salivarius* and *S. sobrinus* (condition E), although it was not significant.

The pH values for *S. sobrinus* in dual species model with *S. salivarius* were determined (Table 6).

**Table 6: pH values for *Streptococcus sobrinus* in dual species model at 24 h and 48 h:** Assessment of pH variation of *S. sobrinus* culture testing the effect of C7 compound with (E) or without (D) the presence of *S. salivarius* and glucose with (C) or without (B) *S. salivarius*. The negative controls were made one with only the cariogenic bacterium (A) and another with the cariogenic bacterium and *S. salivarius* without addition of glucose (F)

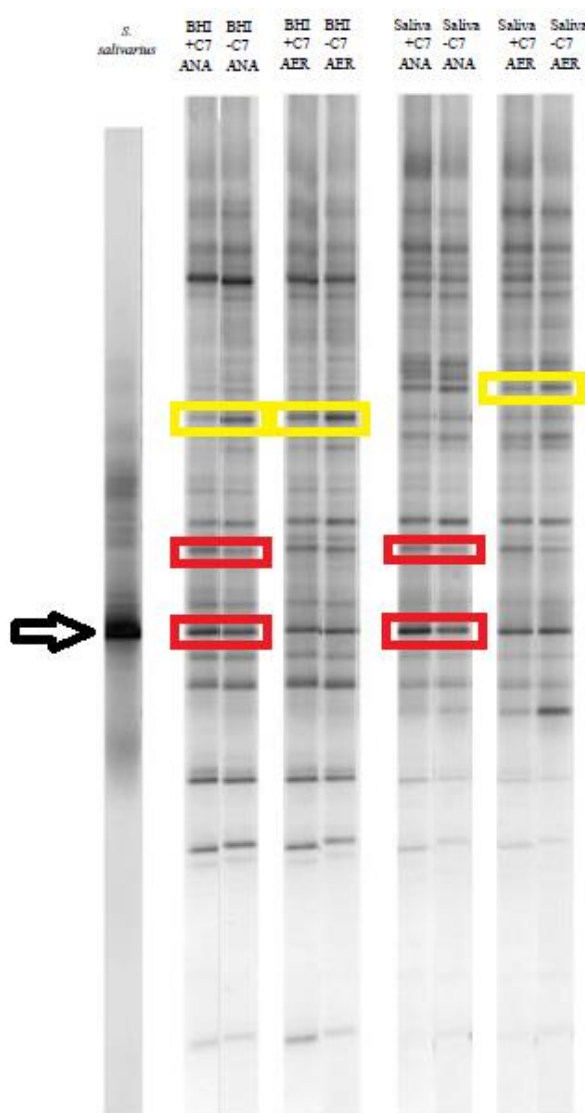
Condition	pH values					
	A	B	C	D	E	F
24 h	5.76	5.06	4.95	5.64	4.87	5.60
48 h	5.78	5.08	4.99	5.64	4.89	5.64

An average pH of 5.76 was detected in the control (condition A) when looking after 24 h of the start of the experiment. A lower pH than the control was observed when *S. sobrinus* is present with glucose (condition B), glucose and *S. salivarius* (condition C), and *S. salivarius* and C7 compound (condition E). The pH was higher in condition B and the difference was greater between conditions B and E than between B and C. The lowest values are observed when *S. salivarius* is present together with glucose (condition C) or the C7 compound (condition E).

However, when *S. salivarius* was present together with *S. sobrinus* pH was higher. So, when glucose or the C7 compound was present, an extra decrease can be seen. The values after 48 h are approximately the same and there were no variations observed (Table 6).

### 3.3.3. Effect of a prebiotic compound on bacteria present in saliva

A PCR-DGGE analysis was performed in order to understand the effect of a prebiotic compound (C7) on the whole bacterial community present in human saliva and BHI medium was used as a positive control. The results are shown in Figure 14.



**Figure 14:** PCR-DGGE analysis of effect of C7 compound in BHI and saliva after 24 h. BHI: Brain-Heart Infusion. C7: prebiotic compound. ANA: Anaerobic Conditions. AER: Aerobic Conditions. Black arrow: *S. salivarius* marker. Red rectangles: bands more clearly with the presence of C7 compound. Yellow rectangles: bands more clearly without the presence of C7 compound.

*S. salivarius* was used as a mark (black arrow) and some differences were detected according to the presence or not of the prebiotic compound. Comparing the results with and

without the C7 compound it is possible to see some bands more clearly, mostly in anaerobic conditions. These bands appear at the same length of *S. salivarius* fragment. So, it seems that *S. salivarius* is stimulated by C7 compound in BHI and saliva (red rectangles). On the other hand some bands are more pronounced when C7 compound is not present (yellow rectangles). So, an ecological shift is visible in the whole community.

### 3.4. Discussion

According to the ecological plaque hypothesis, an alternative strategy to treat and prevent ecological shift in oral diseases is related to the use of beneficial indigenous bacteria and probiotics stimulated by the use of prebiotics [21]. Probiotics and prebiotics are an alternative to change and prevent ecological shift in oral diseases.

A selective medium is essential to cultivate a specific microorganism, because it allows the growth of certain microorganisms inhibiting others.

Mitis-Salivarius (MS) agar is normally used for *mutans streptococci* isolation [66], [67]. MS was tested for *S. salivarius*, *S. mutans* and *S. sobrinus* (Figure 7-I). As expected, *S. mutans* and *S. sobrinus* grew on this medium [66]. However *S. salivarius* also grew on MS agar plates. It is also not possible to distinguish between *S. mutans* (Figure 7-IB) and *S. sobrinus* (Figure 7-IC), as expected [66], [68]. So, it was concluded that this medium is non-selective neither for the cariogenic strains neither for the beneficial strain. Hirasawa et al. (2003) developed a selective medium for *S. mutans* (MS-MUT) and for *S. sobrinus* (MS-SOB) based on MS agar medium combined with reduced levels of sucrose and several antibiotics (sulfisoxazole, bacitracin, cinoxacin and fleoxacin).

On the other hand, Van Palenstein et al. (1983) developed a selective medium for the main cariogenic bacteria (*S. mutans* and *S. sobrinus*). The medium trypticase, yeast, cysteine (TYC) agar was modified by addition of bacitracin (TYCSB) and seemed to be selective for these bacteria [68]. In this study, it was confirmed that TYCSB allowed the growth of *S. mutans* and *S. sobrinus* (Figure 7-II). *S. salivarius* did not grow on TYCSB plates and this absence of growth is probably due to components of TYCSB medium when compared with MS agar medium. Comparing MS and TYCSB components, a possible explanation is the presence of salts ( $\text{Na}_2\text{SO}_3$ ,  $\text{NaHCO}_3$ ,  $\text{NaCl}$ ,  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  and  $\text{C}_2\text{H}_3\text{O}_2\text{Na} \cdot 3\text{H}_2\text{O}$ ) on TYCSB medium, stimulating bacterial growth. In conclusion, a selective medium (TYCSB) was found for the two cariogenic strains.

However, a selective medium for the beneficial strain still need to be found. A modified minimal medium (MM) was the hypothesis tested (data not shown). A minimal medium contains the minimum nutrients (as carbon source) and essential elements for protein and nucleic acid synthesis by microorganisms. The MM medium used did not allow selective growth of *S. salivarius*, since *S. mutans* and *S. sobrinus* also grew. In conclusion, MM medium could not be used as a selective medium for *S. salivarius*, thereby no selective medium for this beneficial strain was found.

To test whether addition of a prebiotic compound (C7) conjugated with a probiotic strain (*S. salivarius*) on cariogenic bacteria (*S. mutans* and *S. sobrinus*), dual species experiments were carried out. *S. mutans* is a cariogenic bacterium present in high levels in caries [2]. Looking at Figure 8, significant differences were detected in all conditions after 24 h, when compared to control (condition A). Besides these significant differences, conditions with *S. salivarius* (conditions C, E and F) seem to have less growth of *S. mutans*. So, *S. salivarius* seems to have effect on *S. mutans* reduction. This can be explained by the beneficial effect of *S. salivarius* as a probiotic strain [60]. On the other hand, C7 compound seems to not have effect on lesser growth of *S. mutans*, because reduction of *S. mutans* is only verified when C7 is conjugated with *S. salivarius* and not when *S. mutans* is alone with C7. Further, the presence of glucose with *S. mutans* and *S. salivarius* resulted in the lowest growth of the cariogenic bacterium, when compared with the presence of C7 compound, but actually glucose can be metabolized by *S. mutans* [69]. So, glucose seems to stimulate *S. mutans* growth, however cannot be used as a prebiotic compound due its metabolism by this cariogenic bacterium [69]. Thus, the verified lowest increase of *S. mutans* are probably due to the presence of *S. salivarius* and not because of presence of C7 compound.

After 48 h, all conditions presented a reduction in relation to 24h. All conditions with *S. salivarius* significantly decreased when compared with the control (*S. mutans* alone). C7 compound seems not to have influence in the verified reduction, however the reduction in the presence of C7 compound is slightly higher than without the compound, showing the enhancement of probiotics by prebiotics, as expected [58]. Further, another significant reduction was verified in the presence of glucose, a carbon source that can be metabolized by *S. mutans* [69]. So, the significant reductions could be probably as a result of the presence of *S. salivarius* and has no variation due to C7 compound.

Another significant difference was verified comparing *S. mutans* and *S. salivarius* with and without glucose. The absence of glucose seems to be more positive, when compared to *S. mutans* and *S. salivarius* which did not is clearly evident, when comparing this condition without supplement to the control.

qPCR was performed for *S. mutans* quantification in dual species experiment (Figure 9). Lesser growth of this cariogenic bacterium can be observed when *S. mutans* is present together with *S. salivarius*, with or without the presence of glucose, when compared to only *S. mutans*. This could mean an influence of *S. salivarius* leading to *S. mutans* inhibition, supporting its effect as a probiotic [60]. However, the presence of C7 compound in *S. mutans* and *S. salivarius* had no significant difference, even conjugated with *S. salivarius*. So, the presence of C7 compound did not influence *S. mutans* growth. The growth of *S. mutans* is little lower when glucose is presence. However, glucose cannot be considered as a prebiotic in this case because it is metabolized by *S. mutans* [69]. In conclusion, the reduced growth of *S. mutans* after 24 h was probably motivated by the presence of *S. salivarius* and not because of presence of C7 compound.

Looking at 48 h, no significant reductions were observed and the values obtained by qPCR are also counting dead cells, which constitutes a limitation of current biological technologies for quantification of specific cells and/or strains in mixed samples [70]. Thus, certainly some conditions had an increase in concentration resulting from dead cells counting by qPCR.

Comparing microbial culturing and qPCR for *S. mutans* in dual species experiment, the presence of *S. salivarius* seems to have reducing effect on *S. mutans* and the presence of C7 compound does not have influence on it. Further, the presence of glucose also stimulates the reduction of *S. mutans*, however glucose cannot be used as a prebiotic. The values obtained were almost the same although the higher values for qPCR for 48 h, when compared with microbial culturing, probably due to the count of dead cells.

Behind the analysis of cariogenic bacterium concentration due to the presence of glucose or C7 compound, it is important to take a look in the behavior of the probiotic strain used. Thus, looking at Figure 10, a significant increase was verified when *S. salivarius* is present together with *S. mutans*. This can corroborate the hypothesis of *S. salivarius* as an important oral probiotic [2], [60]. C7 compound seems to not have influence on *S. salivarius* growth.



Comparing *S. mutans* (Figure 9) and *S. salivarius* (Figure 10) concentrations in *S. mutans* dual species experiment, it was concluded that the presence of *S. salivarius* could leave to a reduction of *S. mutans*.

The effect of pH in dual species experiments was tested because a low pH can be the result of acid production by oral *streptococci* due to sugar intake and later to demineralization [69], [71].

The pH of different conditions tested did not suffer variations and the values were maintained almost constant after 24 h and after 48 h (Table 5). However, all of them can be characterized by an acidic environment. Although no significant differences were detected, it is important to analyze pH values obtained for the different conditions. A higher decrease in pH was verified when glucose or C7 compound is combined with *S. salivarius*, compared with control with only *S. mutans*, which could mean the influence of these supplements, separately, in pH reduction.

Analyzing the conditions of *S. mutans* where C7 is present, when *S. salivarius* is present, the pH (4.8) falls. However when *S. mutans* is only present with C7, pH value ( $\approx 5.6$ ) is similar to the control ( $\approx 5.6$ ). When *S. mutans* is conjugated only with *S. salivarius* (condition F), pH is almost around 5.4, nearly to the value obtained when *S. mutans* was alone (condition A).

Similar dual species experiments were also carried out to see the effect of prebiotic compound used in this study in another cariogenic bacterium, *S. sobrinus* (Figure 11). After 24 h, *S. salivarius* seems to have an effect on *S. sobrinus*, but not a significant effect. However, compared to 0 h, these conditions had a non-significant decrease when conjugated with a carbon source (glucose or C7 compound).

On the other hand, a large and significant increase was verified when *S. sobrinus* is present together with C7 compound after 24 h. This could be associated to unculturable cells present [72]. The viable but non-culturable cells (VBNC) are still with metabolic activity but cannot be detected using classical cultivation on agar substrates solid media [73]. Further, in this state bacteria are able to resuscitate and become culturable again, but still metabolically inactive [63], [74]. VBNC can be a result of different situations such as natural stress, extremes of temperature, pH, oxygen concentration [63], [74].

After 48 h, all conditions suffered a reduction on *S. sobrinus*, maybe due to the lack of nutrients for growth, which seems to be more accentuated when *S. sobrinus* is alone (condition A), probably due to VBNC state as a consequence of lack of the required nutrients [74].

So, it was concluded that there is no effect of the C7 compound, only a small effect of presence of *S. salivarius* but not really clear.

Regarding to qPCR of *S. sobrinus* in dual species experiment after 24 h (Figure 12), glucose has no influence on *S. sobrinus* reduction. On the other hand, the presence of *S. salivarius* leads to a lesser growth of *S. sobrinus*, both with and without the presence of C7 compound, which is significant in the presence of this compound. The presence of *S. salivarius* seems to stimulate a lesser growth of *S. sobrinus*, although the little differences, when compared to other conditions. Once again, *S. salivarius* appears with probiotic potential [60]. So, it was concluded that the presence of *S. salivarius* has influence on *S. sobrinus* and the presence of C7 compound seems to be positive for it.

As explained before, qPCR cannot distinguish between live and dead cells [55]. So, some verified increases can be due to this limitation of qPCR method.

Comparing microbial culturing (Figure 11) and qPCR (Figure 12) for *S. sobrinus* in dual species experiment, the presence of *S. salivarius* seems to have reducing effect on *S. sobrinus* with influence by the presence of C7 compound. This was not really clear in microbial culturing maybe due to viable but non-culturable cells [63]. Further, the large increase verified on *S. sobrinus* when is conjugated only with C7 compound after 24 h in microbial culturing is not observed in qPCR.

Regarding to the variation of *S. salivarius* in dual species experiment of *S. sobrinus* by qPCR, a significant increase was detected when *S. salivarius* is present together with *S. sobrinus*, highlighting the effect of this probiotic strain, already tested [60]. This was verified in comparison with presence of C7 compound. So, this corroborates the effect of *S. salivarius* in *S. sobrinus* that was already verified by qPCR for *S. sobrinus* in Figure 12.

Comparing values of *S. sobrinus* (Figure 12) with *S. salivarius* (Figure 13) the presence of *S. salivarius* together with *S. sobrinus* caused a little reduction on this cariogenic bacterium due to an increase of *S. salivarius*, which however seems to be stimulated by C7 compound.

The pH values for *S. sobrinus* maintained their values through time, depending on conditions considered (Table 6). However, pH fall was verified for conditions with *S. sobrinus* and glucose ( $\approx 5$ ), and glucose and *S. salivarius* ( $\approx 5$ ). The other pH fall, when compared with only *S. sobrinus*, was verified when *S. sobrinus* was conjugated with *S. salivarius* and C7 compound (4.9). So, without addition of glucose or C7 compound, but with addition of only *S. salivarius* (5.6), no decrease of pH was verified.

The PCR-DGGE is a molecular fingerprinting method against 16S rRNA that was used to examine the effect of C7 compound in BHI and saliva after 24 h [75]. For this analysis, saliva samples were collected from healthy people. DGGE allows the analysis of unculturable species present in saliva in which PCR product are separated based on nucleotide composition. Looking at Figure 14, the addition of C7 compound altered the composition of samples and differences between conditions are evident. *S. salivarius* band is more clearly marked in the presence of this prebiotic compound, mainly under anaerobic conditions. This means that in the presence of C7 compound there is an increase of the probiotic strain in saliva meaning that C7 compound stimulates *S. salivarius*. It is known that prebiotics enhance the growth of beneficial bacteria as *lactobacilli* and *bifidobacteria* [52].

With these results, it can be hypothesized that C7 compound stimulates also beneficial *streptococci* as *S. salivarius*, a beneficial bacterium already present in the mouth. Further, other strains have their bands more evidence in the presence of C7 compound. On the other hand, some bands are more evident without the presence of C7 compound, marked as yellow rectangles in Figure 14. In generally, it can be concluded that under anaerobic conditions presence of *S. salivarius* was more evident when conjugated with C7 compound and seems to be stimulated by this prebiotic compound. So, it seems that exists an ecological shift when C7 is or is not present that leads some bands to be more or less marked according to it.

In conclusion, C7 compound seems to influence the presence of *S. salivarius* in saliva sample. Saliva is a complex fluid where several bacteria present in the mouth grow together. So, the results about the effect of a prebiotic compound in dual species experiment concluded that *S. salivarius* has more influence on cariogenic bacteria reduction than the C7 compound. Conjugating the experiments of saliva with the cariogenic bacteria, it could be hypothesized that the effect of C7 compound stimulates an ecological shift to more presence of *S. salivarius*. On the other hand, *S. salivarius* has a positive effect on cariogenic bacteria reduction. So, C7 compound seems to be involved in dental caries etiological bacteria reduction by the effect of *S. salivarius*.

### 3.5. Conclusion

TYCSB was the selective medium used for *S. mutans* and for *S. sobrinus*. However, a selective medium for the probiotic strain used was not found.

*S. salivarius*, the probiotic strain used in this study, seems to have a reduction effect on *S. mutans*. However, C7, the prebiotic used, does not influence this reduction. When glucose is conjugated with *S. salivarius*, *S. mutans* also suffers a reduction, however glucose cannot be used as a prebiotic. The results of qPCR confirmed the influence of *S. salivarius* on *S. mutans* reduction.

For *S. sobrinus*, the prebiotic compound seems to have influence in a little reduction when the cariogenic bacterium is conjugated with the probiotic strain, however it was not really clear.

On the other hand, analyzing a PCR-DGGE of saliva, the prebiotic seems to stimulate a microbial shift, evidencing *S. salivarius*, when the prebiotic is present. Other shifts were verified for other strains meaning a shift in the whole community in the mouth.

In conclusion, the prebiotic compound seems to influence the presence of *S. salivarius* on saliva. Dual species experiments concluded that apparently *S. salivarius* have influence on *S. mutans* reduction and not really clear on *S. sobrinus*. In conclusion, C7 compound has a positive effect on *S. salivarius*, which by turn stimulates cariogenic bacteria reduction.



## CHAPTER 4

### Conclusion and future work



The present work presented an application, for the first time, of the technique of quantitative Polymerase Chain Reaction (qPCR) for quantification of *Streptococcus salivarius*. Moreover, it was also aim to study the effect of a prebiotic compound (C7) on cariogenic bacteria and also on oral flora present in saliva, in order to understand whether addition of this compound leads to a decrease of these bacteria (*Streptococcus mutans* and *Streptococcus sobrinus*). Additionally, the effect of a probiotic species (*Streptococcus salivarius*) present in the mouth, was also tested.

The enumeration by qPCR presented a linear relationship with the microbial culturing of *S. salivarius*. So, this new method for this probiotic strain allowed its quantification, eliminating gel electrophoresis analysis.

Regarding the dual species experiments, a selective medium for *S. mutans*, *S. sobrinus* and *S. salivarius* was searched. TYCSB medium was the selective medium used in this study for the cariogenic bacteria. However no selective medium was found for the probiotic strain.

Analyzing the effect of the prebiotic compound (C7) in cariogenic bacteria reduction, no clear conclusions were possible to obtain. For *S. mutans*, the major influence in its reduction was probably due to the probiotic strain (*S. salivarius*) but there was no influence of C7. For *S. sobrinus*, there was some reduction in the presence of *S. salivarius* with stimulation of the prebiotic compound (C7), however this was not completely clear with the experiments performed.

On the other hand, it was shown that C7 had an effect in the oral microbiota present in human saliva, since it was verified an ecological shift in the whole community. *S. salivarius* seems to be more present when C7 is added to saliva. On the other hand, other species are in higher amounts in saliva when C7 is not present.

As future work, it would be interesting to find a selective medium for *S. salivarius* in order to quantify by microbial culturing this probiotic strain in a dual species model experiment with cariogenic bacteria.

Another suggestion is related to other energy sources, other compounds or substrates that should be found in order to explore the relation and stimulation in beneficial and pathogenic bacteria present in the mouth and involved in dental caries.





## CHAPTER 5

### References



- [1] K. Hojo, S. Nagaoka, T. Ohshima, and N. Maeda, "Bacterial interactions in dental biofilm development," *J. Dent. Res.*, vol. 88, no. 11, pp. 982–990, Nov. 2009.
- [2] R. Huang, M. Li, and R. L. Gregory, "Bacterial interactions in dental biofilm," *Virulence*, vol. 2, no. 5, pp. 435–444, 2011.
- [3] W. Teughels, N. Van Assche, I. Sliepen, and M. Quirynen, "Effect of material characteristics and/or surface topography on biofilm development," *Clin. Oral Implants Res.*, vol. 17 Suppl 2, pp. 68–81, 2006.
- [4] W. Teughels, G. Loozen, and M. Quirynen, "Do probiotics offer opportunities to manipulate the periodontal oral microbiota?," *J. Clin. Periodontol.*, vol. 38 Suppl 1, pp. 159–77, Mar. 2011.
- [5] W. Teughels, M. Van Essche, I. Sliepen, and M. Quirynen, "Probiotics and oral healthcare," *Periodontol. 2000*, vol. 48, pp. 111–147, Jan. 2008.
- [6] A. R. Ten Cate, "Structure of the Oral Tissues," in in *Oral Histology - Development, Structure and Function*, 1998, pp. 1–9.
- [7] P. D. Marsh, "Microbial Ecology of Dental Plaque and its Significance in Health and Disease," *Adv. Dent. Res.*, vol. 8, no. 2, pp. 263–271, 1994.
- [8] P. D. Marsh and B. Nyvad, "The oral microflora and biofilms on teeth," in in *Dental Caries - The Disease and its Clinical Management*, 2008, pp. 163–187.
- [9] N. Suzuki, A. Yoshida, and Y. Nakano, "Quantitative Analysis of Multi-Species Oral Biofilms by TaqMan Real-Time PCR," *Clin. Med. Res.*, vol. 3, no. 3, pp. 176–185, 2005.
- [10] P. D. Marsh, "Are dental diseases examples of ecological catastrophes?," *Microbiology*, vol. 149, no. 2, pp. 279–294, Feb. 2003.
- [11] C. A. Squier and M. W. Finkelstein, "Oral Mucosa," in in *Oral Histology - Development, Structure and Function*, 1998, pp. 345–385.
- [12] M. Jawed, S. M. Shahid, S. A. Qader, and A. Azhar, "Dental caries in diabetes mellitus: role of salivary flow rate and minerals," *J. Diabetes Complications*, vol. 25, no. 3, pp. 183–186, 2011.
- [13] M. J. Verkaik, "Oral Biofilms - Mechanical and Chemical Control Measures," Rijksuniversiteit Groningen, 2011.
- [14] F. A. Roberts and R. P. Darveau, "Beneficial bacteria of the periodontium," *Periodontol. 2000*, vol. 30, pp. 40–50, Jan. 2002.
- [15] Z. T. Wen and R. A. Burne, "Functional Genomics Approach to Identifying Genes Required for Biofilm Development by *Streptococcus mutans*," *Appl. Environ. Microbiol.*, vol. 68, no. 3, pp. 1196–1203, 2002.

- [16] A. F. P. Leme, H. Koo, C. M. Bellato, G. Bedi, and J. A. Cury, "The Role of Sucrose in Cariogenic Dental Biofilm Formation - New Insight," *J. Dent. Res.*, vol. 85, no. 10, pp. 878–887, Oct. 2006.
- [17] Z. T. Wen, D. Yates, S.-J. Ahn, and R. A. Burne, "Biofilm formation and virulence expression by *Streptococcus mutans* are altered when grown in dual-species model," *BMC Microbiol.*, vol. 10, no. 1, p. 111, 2010.
- [18] N. Takahashi and B. Nyvad, "Caries Ecology Revisited: Microbial Dynamics and the Caries Process," *Caries Res.*, vol. 42, no. 6, pp. 409–418, Jan. 2008.
- [19] P. D. Marsh, "Dental plaque as a biofilm and a microbial community - implications for health and disease," *BMC Oral Health*, vol. 6 Suppl 1, p. S14, Jan. 2006.
- [20] N. Takahashi and B. Nyvad, "The Role of Bacteria in the Caries Process: Ecological Perspectives," *J. Dent. Res.*, vol. 90, no. 3, pp. 294–303, 2011.
- [21] D. A. Devine and P. D. Marsh, "Prospects for the development of probiotics and prebiotics for oral applications," *J. Oral Microbiol.*, vol. 1, no. 6, pp. 1–11, Jan. 2009.
- [22] A. Bhardwaj and S. V. Bhardwaj, "Periodontitis as a risk factor for cardiovascular disease with its treatment modalities: a review," *J. Mol. Pathophysiol.*, vol. 1, no. 1, pp. 77–83, 2012.
- [23] C. G. Van Hoogmoed, G. I. Geertsema-doornbusch, W. Teughels, M. Quirynen, H. J. Busscher, and H. C. Van der Mei, "Reduction of periodontal pathogens adhesion by antagonistic strains," *Oral Microbiol. Immunol.*, vol. 23, pp. 43–48, 2008.
- [24] S. Kothari, G. Gnanarajan, and P. Kothiyal, "Periodontal chip: an adjunct to conventional surgical treatment," *Int. J. Drug Res. Technol.*, vol. 2, no. 6, pp. 411–421, 2012.
- [25] I. Sliepen, M. Van Essche, M. Pauwels, J. Van Eldere, J. Hofkens, M. Quirynen, and W. Teughels, "Colonization of hard and soft surfaces by *Aggregatibacter actinomycetemcomitans* under hydrodynamic conditions," *Oral Microbiol. Immunol.*, vol. 23, pp. 498–504, 2008.
- [26] A. Azarpazhooh, P. S. Shah, H. C. Tenenbaum, and M. B. Goldberg, "The Effect of Photodynamic Therapy for Periodontitis: A Systematic Review and Meta-Analysis," *J. Periodontol.*, vol. 81, no. 1, pp. 4–14, 2009.
- [27] W. Teughels, M. G. Newman, W. Coucke, A. D. Haffajee, H. C. Van Der Mei, S. Kinder Haake, E. Schepers, J.-J. Cassiman, J. Van Eldere, D. van Steenberghe, and M. Quirynen, "Guiding Periodontal Pocket Recolonization: a Proof of Concept," *J. Dent. Res.*, vol. 86, no. 11, pp. 1078–1082, 2007.
- [28] I. Sliepen, M. Van Essche, G. Loozen, J. Van Eldere, M. Quirynen, and W. Teughels, "Interference with *Aggregatibacter actinomycetemcomitans*: colonization of epithelial cells under hydrodynamic conditions," *Oral Microbiol. Immunol.*, vol. 24, pp. 390–395, 2009.

- [29] M. Van Essche, M. Quirynen, I. Sliepen, G. Loozen, N. Boon, J. Van Eldere, and W. Teughels, "Killing of anaerobic pathogens by predatory bacteria," *Mol. Oral Microbiol.*, vol. 26, pp. 52–61, 2011.
- [30] A. Sheiham, "Dietary effects on dental diseases," *Public Health Nutr.*, vol. 4, no. 2B, pp. 569–591, Sep. 2001.
- [31] R. J. Gibbons and J. van Houte, "Dental caries," in in *Annual Review of Medicine*, vol. 26, 1975, pp. 122–136.
- [32] T. Oho, Y. Yamashita, Y. Shimazaki, M. Kushiya, and T. Koga, "Simple and rapid detection of *Streptococcus mutans* and *Streptococcus sobrinus* in human saliva by polymerase chain reaction," *Oral Microbiol. Immunol.*, vol. 15, no. 4, pp. 258–262, Aug. 2000.
- [33] A. R. Ten Cate, "Repair and Regeneration of Dental Tissue," in in *Oral Histology - Development, Structure and Function*, 1998, pp. 408–423.
- [34] E. Freeman, "Periodontium," in in *Oral Histology - Development, Structure and Function*, 1998, pp. 253–288.
- [35] N. Takahashi, "Microbial ecosystem in the oral cavity: Metabolic diversity in an ecological niche and its relationship with oral diseases," *Int. Congr. Ser.*, vol. 1284, pp. 103–112, Sep. 2005.
- [36] B. Brito Bezerra, O. Andriankaja, J. Kang, S. Pacios, H. Jin Bae, Y. Li, V. Tsiagbe, H. Schreiner, D. H. Fine, and D. T. Graves, "A *actinomycetemcomitans*-induced periodontal disease promotes systemic and local responses in rat periodontium," *J. Clin. Periodontol.*, vol. 39, pp. 333–341, 2012.
- [37] J. Travis, R. Pike, T. Imamura, and J. Potempa, "Porphyromonas gingivalis proteinases as virulence factors in the development of periodontitis," *J. Periodontal Res.*, vol. 32, pp. 120–125, 1997.
- [38] G. M. Zanatta, S. Bittencourt, F. H. Nociti Jr., E. A. Sallum, A. W. Sallum, and M. Z. Casati, "Periodontal Debridement With Povidone-Iodine in Periodontal and Biochemical Observations," *J. Periodontol.*, vol. 77, no. 3, pp. 498–505, 2006.
- [39] A. Yoshida, N. Suzuki, Y. Nakano, M. Kawada, T. Oho, and T. Koga, "Development of a 5' Nuclease-Based Real-Time PCR Assay for Quantitative Detection of Cariogenic Dental Pathogens *Streptococcus mutans* and *Streptococcus sobrinus*," *J. Clin. Microbiol.*, vol. 41, no. 9, pp. 4438–4441, 2003.
- [40] O. Fejerskov, B. Nyvad, and E. A. M. Kidd, "Pathology of dental caries," in in *Dental Caries - The Disease and its Clinical Management*, 2008, pp. 19–48.

- [41] S. Twetman, S. Axelsson, G. Dahlén, I. Espelid, I. Mejàre, A. Norlund, and S. Tranæus, "Adjunct methods for caries detection: a systematic review of literature," *Acta Odontologica Scandinavica*, vol. 71, no. 3–4, pp. 1–10, 2012.
- [42] O. Fejerskov, E. A. M. Kidd, B. Nyvad, and V. Baelum, "Defining the disease: an introduction," in in *Dental Caries - The Disease and its Clinical Management*, 2008, pp. 3–6.
- [43] O. Fejerskov, B. Nyvad, and E. A. M. Kidd, "Clinical appearances of caries lesions," in in *Dental Caries - The Disease and its Clinical Management*, 2008, pp. 7–18.
- [44] B. A. Burt, V. Baelum, and O. Fejerskov, "The epidemiology of dental caries," in in *Dental Caries - The Disease and its Clinical Management*, 2008, pp. 123–145.
- [45] A. Yano, N. Kaneko, H. Ida, T. Yamaguchi, and N. Hanada, "Real-time PCR for quantification of *Streptococcus mutans*," *FEMS Microbiol. Lett.*, vol. 217, pp. 23–30, 2002.
- [46] J. H. Meurman and I. Stamatova, "Probiotics: contributions to oral health," *Oral Dis.*, vol. 13, no. 5, pp. 443–51, Sep. 2007.
- [47] J. H. Meurman, "Probiotics: do they have a role in oral medicine and dentistry?," *Eur. J. Oral Sci.*, vol. 113, no. 3, pp. 188–196, Jun. 2005.
- [48] G. Reid, "Probiotics and prebiotics – Progress and challenges," *Int. Dairy J.*, vol. 18, no. 10–11, pp. 969–975, Oct. 2008.
- [49] A. Homayoni Rad, F. Akbarzadeh, and E. V. Mehrabany, "Which are more important: Prebiotics or probiotics?," *Nutrition*, vol. 28, no. 11–12, pp. 1196–1197, 2012.
- [50] J. Schrezenmeir and M. de Vrese, "Probiotics, prebiotics, and synbiotics—approaching a definition," *Am. J. Clin. Nutr.*, vol. 73, no. 2 Suppl, p. 361S–364S, Feb. 2001.
- [51] T. S. Manning and G. R. Gibson, "Prebiotics," *Best Pract. Res. Clin. Gastroenterol.*, vol. 18, no. 2, pp. 287–298, 2004.
- [52] M. Roberfroid, "Prebiotics: The Concept revisited," *J. Nutr.*, vol. 137, no. 3 Suppl 2, p. 830S–837S, Mar. 2007.
- [53] M. Van Essche, I. Sliepen, G. Loozen, J. Van Eldere, M. Quirynen, Y. Davidov, E. Jurkevitch, N. Boon, and W. Teughels, "Development and performance of a quantitative PCR for the enumeration of *Bdellovibrionaceae*," *Environ. Microbiol. Rep.*, vol. 1, no. 4, pp. 228–233, Aug. 2009.
- [54] T. Igarashi, Y. Yano, A. Yamamoto, R. Sasa, and N. Goto, "Identification of *Streptococcus salivarius* by PCR and DNA probe," *Let. Appl. Microbiol.*, vol. 32, pp. 394–397, 2001.

- [55] G. Loozen, N. Boon, M. Pauwels, M. Quirynen, and W. Teughels, "Live/dead real-time polymerase chain reaction to assess new therapies against dental plaque-related pathologies.," *Mol. Oral Microbiol.*, vol. 26, no. 4, pp. 253–61, Aug. 2011.
- [56] Brookman-Amissah, Ha. Packer, E. Prediger, and J. Sabel, *qPCR Application Guide*. 2012.
- [57] J. Hellemans, G. Mortier, A. De Paepe, F. Speleman, and J. Vandesompele, "qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data," *Genome Biol.*, vol. 8, no. 2, p. R19, Jan. 2007.
- [58] B. M. Corcoran, R. P. Ross, G. F. Fitzgerald, and C. Stanton, "Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances," *J. Appl. Microbiol.*, vol. 96, no. 5, pp. 1024–1039, Jan. 2004.
- [59] P. M. Giffard, C. L. Simpson, C. P. Milward, and N. A. Jacques, "Molecular characterization of a cluster of at least two glucosyltransferase genes in *Streptococcus salivarius* ATCC 25975," *J. Gen. Microbiol.*, vol. 137, no. 11, pp. 2577–2593, Nov. 1991.
- [60] J. P. Burton, P. A. Wescombe, C. J. Moore, C. N. Chilcott, and J. R. Tagg, "Safety Assessment of the Oral Cavity Probiotic *Streptococcus salivarius* K12," *Appl. Environ. Microbiol.*, vol. 72, no. 4, pp. 3050–3053, 2006.
- [61] J. R. Cole, B. Chai, R. J. Farris, Q. Wang, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, A. M. Bandela, E. Cardenas, G. M. Garrity, and J. M. Tiedje, "The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data," *Nucleic Acids Res.*, vol. 35, pp. D169–172, 2007.
- [62] R. S. Reddy, L. A. Swapna, T. Ramesh, T. R. Singh, N. Vijayalaxmi, and R. Lavanya, "Bacteria in Oral Health – Probiotics and Prebiotics A Review," *Int. J. Biol. Med. Res.*, vol. 2, no. 4, pp. 1226–1233, 2011.
- [63] J. D. Oliver, "The Viable but Nonculturable State in Bacteria," *J. Microbiol.*, vol. 43, no. Special issue, pp. 93–100, 2005.
- [64] Y. Li, D. Saxena, V. M. Barnes, H. M. Trivedi, Y. Ge, and T. Xu, "Polymerase chain reaction-based denaturing gradient gel electrophoresis in the evaluation of oral microbiota.," *Oral Microbiol. Immunol.*, vol. 21, no. 5, pp. 333–9, Oct. 2006.
- [65] G. Muyzer, E. C. de Waal, and A. G. Uitterlinden, "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA," *Appl. Environ. Microbiol.*, vol. 59, no. 3, pp. 695–700, 1993.
- [66] M. Hirasawa and K. Takada, "A New Selective Medium for *Streptococcus mutans* and the Distribution of *S. mutans* and *S. sobrinus* and Their Serotypes in Dental Plaque," *Caries Res.*, vol. 37, no. 3, pp. 212–217, 2003.



- [67] O. G. Gold, H. V. Jordan, and J. Van Houte, "A selective medium for *Streptococcus mutans*," *Arch. Oral Biol.*, vol. 18, pp. 1357–1364, 1973.
- [68] W. H. Van Palenstein Helderman, M. Ijsseldijk, and J. H. Huis In 't Veld, "A selective medium for the two major subgroups of the bacterium *Streptococcus mutans* isolated from human dental plaque and saliva.," *Arch. Oral Biol.*, vol. 28, no. 7, pp. 599–603, Jan. 1983.
- [69] S. M. Colby and R. R. B. Russell, "Sugar metabolism by *mutans streptococci*," *J. Appl. Microbiol. Symp. Suppl.*, vol. 83, p. 80S–88S, Jan. 1997.
- [70] K. Rudi, B. Moen, S. M. Drømtorp, and A. L. Holck, "Use of Ethidium Monoazide and PCR in Combination for Quantification of Viable and Dead Cells in Complex Samples," *Appl. Environ. Microbiol.*, vol. 71, no. 2, pp. 1018–1024, 2005.
- [71] Y.-H. Li, M. N. Hanna, G. Svensäter, R. P. Ellen, and D. G. Cvitkovitch, "Cell Density Modulates Acid Adaptation in *Streptococcus mutans*: Implications for Survival in Biofilms," *J. Bacteriol.*, vol. 183, no. 23, pp. 6875–6884, 2001.
- [72] C. A. Fux, J. W. Costerton, P. S. Stewart, and P. Stoodley, "Survival strategies of infectious biofilms," *Elsevier*, vol. 13, no. 1, pp. 34–40, Jan. 2005.
- [73] E.-M. Decker, "The ability of direct fluorescence-based, two-colour assays to detect different physiological states of oral streptococci," *Lett. Appl. Microbiol.*, vol. 33, no. 3, pp. 188–192, Sep. 2001.
- [74] J. T. Trevors, "Viable but non-culturable (VBNC) bacteria: Gene expression in planktonic and biofilm cells," *J. Microbiol. Methods*, vol. 86, no. 2, pp. 266–73, Aug. 2011.
- [75] G. Muyzer and K. Smalla, "Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology," *Antonie van Leeuwenhoek Acad. Publ. van Leeuwenhoek*, vol. 73, no. 1, pp. 127–141, Jan. 1998.